Universidade de Aveiro Departamento de Biologia
2008

Joana Luísa Lourenço Estevinho Pereira

Variações populacionais de cladóceros sujeitos a diferentes condições de *stress*

Universidade de Aveiro Departamento de Biologia
2008

Joana Luísa Lourenço Estevinho Pereira

Variações populacionais de cladóceros sujeitos a diferentes condições de *stress*

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Fernando José Mendes Gonçalves, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro

Apoio financeiro da Fundação para a Ciência e Tecnologia e do Fundo Social Europeu no âmbito do III Quadro Comunitário de Apoio e por fundos nacionais do Ministério da Ciência, Tecnologia e Ensino Superior – Bolsa de Doutoramento com a referência SFRH/BD/13682/2003.

Aos meus pais!
e a todos aqueles que nunca se resignam com aquilo que já sabem, e que cultivam a “ciência” de aprender, todos os dias…

o júri

presidente

Prof. Doutor Helmuth Robert Malonek
professor catedrático da Universidade de Aveiro

Prof. Doutor Amadeu Mortágua Velho da Maia Soares
professor catedrático da Universidade de Aveiro

Prof. Doutor Vítor Manuel de Oliveira e Vasconcelos
professor associado com agregação da Faculdade de Ciências da Universidade do Porto

Prof. Doutor Carlos Manuel Marques Palmeira
professor associado da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Prof. Doutor Fernando José Mendes Gonçalves (orientador)
professor auxiliar com agregação da Universidade de Aveiro

Prof. Doutor Mário Jorge Verde Pereira
professor auxiliar da Universidade de Aveiro

Prof. Doutora Ruth Maria de Oliveira Pereira
Investigadora auxiliar do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

agradecimentos

Ao meu orientador científico, Prof. Doutor Fernando Gonçalves, agradeço todo o apoio científico fundamental no desenvolvimento deste trabalho. Não posso esquecer o facto de me ter integrado na sua equipa de investigação, oferecendo-me a oportunidade de viver um desafio profissional e pessoal ímpar. Estou ainda grata pela plasticidade com que encarou novas ideias e apoiou novos rumos, ainda que eles fossem algo imprevisíveis. Espero ter conseguido de alguma forma retribuir o investimento.

Aos colegas do LEAD que acompanharam de perto o meu percurso, Bruno, Catarina, Nelson, Ruth, Sara e Sérgio. A vocês devo o que consigo ser hoje dentro de um laboratório; foram vocês os responsáveis por grande parte da minha formação técnica, foram quem muitas vezes me deu ânimo, e foram também quem me ensinou muito do que eu precisava saber, em vários domínios, para desenvolver este trabalho. À Catarina e ao Nelson estou grata por todo o companheirismo, pela boa disposição de todo o dia e pelas descompensações alegres dos fins de dia, por toda a ajuda e apoio; e ao Bruno agradeço a disponibilidade para as frutíferas conversas e discussões sobre tudo e sobre Ciência que temos tido, por toda ajuda, apoio e confiança que me concedeu, e pela sua postura de navegante eternamente insatisfeito. Deixo ainda um agradecimento especial à Cristiana, à Patrícia, à Cláudia – presenças importantes e espíritos bonitos em espaços e momentos distintos – e devo ainda lembrar a Ana Marta, a Ana Sofia, a Inês, a Joana V., o Joaquim, a Márcia, o Nuno M.; são ou foram colegas que, de diversas formas, contribuíram para a minha formação (pessoal e/ou profissional).

Aos meus amigos. Especialmente à Joana e à Xana porque mesmo quando estão longe estão sempre muito perto; à Caty e ao Néné pelas gargalhadas e palhaçadas, pelos três estarolas, por saberem tão bem ser meus amigos, por poder contar convosco sempre; à Milla pela presença sempre bem disposta e amiga; À Aurora e à Cláudia que tantas vezes foram o meu grupo de viagem terapêutico; e à minha Fany, companheira sólida das minhas pequenas loucuras, das vitórias e das minhas maiores tristezas. Estiveram comigo incondicionalmente e ajudaram-me a manter um nível minimamente adequado de lucidez... Muito Obrigada!

Ao Pedro. Obrigada por fazeres parte da minha vida. Obrigada por todos os momentos bons e até pelos momentos menos bons, que me fortaleceram, e pelos momentos em que estiveste presente e em que impediste um ou outro desmoronamento. Obrigada por todas as gargalhadas que demos e damos juntos; por saberes tão bem gozar a vida e com a vida, e por partilhares essa sabedoria comigo, (re)colocando-me as asas que tantas vezes eu insisto em arrumar no recôndito lugar do esquecimento.

À minha família, mas sobretudo àqueles a quem devo tudo o que sou e quem sou, os meus pais. Obrigada por insistirem em ser meus fãs declarados, por serem o meu maior incentivo, por estarem sempre presentes, por chorarem as minhas mágoas comigo, pela cumplicidade por vezes silenciosa mas sempre sábia, por todo o apoio, por saberem tão bem ser estrelas-guia sem guiarem demasiado de perto, pelo carinho com que muitas vezes, mesmo sem saberem, me ajudaram. Obrigada por Tudo!

palavras-chave

Daphnia, estímulos de *stress* naturais e antropogénicos, interação entre estímulos de *stress*, disponibilidade de recursos alimentares, pesticidas, parâmetros individuais, populacionais e sub-celulares, imobilização, expressão genética.

resumo

Os cladóceros, e considerando em particular o género *Daphnia*, são zooplanctontes de reconhecida importância na regulação do equilíbrio dos ecossistemas dulçaquícolas lênticos. Assim, as variações que ocorram em populações naturais destes organismos podem condicionar a eficácia das transferências de energia através da cadeia trófica, eventualmente afectando o biota a diferentes escalas. *Daphnia* é sensível – ao nível individual e ao nível populacional – a diversos estímulos naturais que reflectem as dinâmicas biológicas, físico-químicas ou geo-climáticas associadas ao ecossistema; por outro lado, são também sensíveis a diversos xenobióticos, podendo mesmo ser utilizados para avaliar o potencial destes químicos para afectar as comunidades aquáticas.

A disponibilidade de alimento é, juntamente com a capacidade predatória das comunidades planctívoras, um dos factores bióticos que pode condicionar as populações de *Daphnia*; ainda que seja um tema bastante estudado, os mecanismos que regulam as suas respostas à variação de recursos não são ainda consensuais. Considerando que o tamanho corporal poderia ser um factor regulador, neste trabalho foi avaliado o seu potencial para influenciar a eficiência alimentar de *Daphnia*. Não tendo ficado provada a vantagem competitiva de espécies de maior tamanho corporal, foi possível observar o valor discriminatório do património genético na capacidade de *Daphnia* para explorar os recursos alimentares disponíveis. Sendo assim, a probabilidade da resposta a xenobióticos ser influenciada pelo factor genótipo, condicionando de certa forma a utilização de espécies *standard* na sua avaliação toxicológica, foi assumida como hipótese de trabalho. Foram, portanto, avaliados os efeitos ao nível individual e populacional do herbicida Propanil e do insecticida Metomil, vulgarmente utilizados no controlo de pragas associadas à produção agrícola, em populações de *Daphnia* geneticamente distintas – num desenho experimental que incluiu uma espécie *standard* e três genótipos indígenas – e considerando diferentes cenários de disponibilidade de recursos alimentares. Ambos os pesticidas induziram efeitos deletérios em *Daphnia* sendo que esses efeitos foram moldados de diferentes formas pela abundância de recursos alimentares passíveis de serem explorados pelos organismos.

Nenhum dos pesticidas abordados neste trabalho tem como alvo directo os organismos aquáticos, pelo que o seu modo de acção nestes organismos não está completamente esclarecido. Assim, e considerando ainda que os efeitos observados ao nível do organismo resultam da interacção complexa de efeitos ao nível sub-celular, foram avaliadas as alterações na expressão genética resultantes de exposições agudas a Propanil e a Metomil. Foi registada a activação de diferentes vias metabólicas em resposta a cada um dos tratamentos, afirmando o potencial de pequenas quantidades dos pesticidas na indução de efeitos ao nível sub-celular em *Daphnia*; foi também confirmada a capacidade das ferramentas moleculares de avaliação da expressão genética na discriminação do modo de acção de xenobióticos em organismos não-alvo.

keywords

Daphnia, natural and antropogenic stress stimuli, stressors interaction, resources availability, pesticides, individual, populational and sub-cellular endpoints, imobilisation, life-history, gene expression.

abstract

Cladocerans, and particularly those belonging to the genus *Daphnia*, are widely recognised as key players on the equilibrium regulation in freshwater lentic ecosystems. Variations occurring within *Daphnia* natural populations can constrain the efficiency of the energy transfer along the food web, and eventually promote unexpected large scale changes affecting the biota. Daphnids are sensitive – at the individual and population level – to several natural stimuli, which regard the biological, physico-chemical or geo-climatic dynamics acting on the ecosystem; on the other hand, these organisms are also sensitive to a number of xenobiotics and hence can be used to address the effects of these chemicals in aquatic communities.

Food availability is, along with predatory pressure, one of the biotic factors with greater ability to constrain *Daphnia* populations; the mechanisms behind their responses to resources fluctuation are not fully understood although extensive studies have been carried on this subject. The potential of body size in conditioning *Daphnia* feeding efficiency was evaluated, considering the eventual role of this trait as a regulator in the process. Despite no doubtless evidences were generated on the competitive advantage of large bodied species, genotype was found to be a key factor in discriminating *Daphnia* ability to exploit the available feeding resources. In this way, the value of genotype as a factor conditioning the response to xenobiotics – therefore constraining the single use of standard species in ecotoxicological evaluations – was further hypothesised. The individual- and population-level responses of genetically distinct *Daphnia* populations to commonly applied pesticides (used for the control of several agricultural weeds and pathogens) were addressed. The herbicide Propanil and the insecticide Methomyl were evaluated for their ability to impair the life-history of a standard and three indigenous *Daphnia* genotypes, under different food supply scenarios. Both pesticides induced deleterious effects in *Daphnia*; it should be noticed that, although following different patterns, these effects were actually modulated by resources availability and species exploitation ability.

None of the studied pesticides is specifically designed to kill or damage aquatic organisms, and hence their mode of action in these organisms is not fully known. In this context, and considering that the whole body effects previously observed would be the ultimate outcome of complex interactions between sub-cellular effects, changes in gene expression promoted by exposures to Propanil and Methomyl were evaluated. This experiment showed that each treatment conditioned the activation of different metabolic pathways, and confirmed the potential of small pesticide concentrations in inducing relevant effects at a sub-cellular level in *Daphnia*; the discriminatory potential of gene expression molecular tools for the study of xenobiotics mode of action in non-target organisms was additionally confirmed with this experimental design.

Enquanto não alcançares a verdade não poderás corrigi-la.
Porém se não a corrigires não a alcançarás.
Entretanto não te resignes.

José Saramago

Índice

Capítulo I

Introdução geral

O tema desta dissertação, sendo relativamente lato, converge para um paradigma comum à Ecologia e à Ecotoxicologia: a utilização de organismos-modelo para conhecer os efeitos potenciais de diferentes agentes de *stress* (naturais ou induzidos pela actividade humana) em grupos de organismos com eles relacionados. Numa aplicação mais abrangente, as evidências geradas com alguns destes organismos podem mesmo ser consideradas informação fundamental na modelação e na extrapolação de efeitos prováveis dos agentes de *stress* a diferentes níveis nos ecossistemas. Entre muitos outros organismos, os cladóceros, e em particular os pertencentes ao Género *Daphnia*, são historicamente considerados organismos-modelo em Ecologia, particularmente no contexto das águas doces (*e.g.* Lampert 2006), e em Ecotoxicologia (*e.g.* Baudo 1987, Baird et al. 1989, Sarma & Nandini 2006); mais recentemente, *Daphnia* assumiu também este papel ao nível da Biologia Evolutiva em geral e da Genética de Populações (*e.g.* Hebert 1987, Reznick 1993, Schwenk & Spaak 1995, Deng & Lynch 1996). A extensão em que as características de qualquer organismo-modelo podem ser exploradas em cada uma das áreas dependerá, naturalmente, da qualidade e do detalhe do conhecimento que se detém acerca da biologia desse organismo, por exemplo ao nível da sua ecologia, morfo-fisiologia e sistemática.

Se em teoria qualquer grupo de organismos poderá ser base de trabalho na geração de postulados ou na produção de evidências acerca de teorias e modelos gerais, na prática alguns *taxa* acabam por ser mais apropriados que outros para o efeito. Apesar de haver registos de trabalhos anteriores, a utilização de *Daphnia* como organismo-modelo remontará ao Século XIX (Edmondson 1987) e resulta do reconhecimento das suas características enquanto entidade biológica singular e enquanto entidade ecológica. Os dafnídeos são organismos particularmente interessantes sob o ponto de vista da logística laboratorial, ou seja, são fáceis de cultivar em laboratório porque não exigem grande esforço material e/ou humano para serem mantidos em quantidade e com qualidade suficientes para serem validamente usados em contexto experimental. São organismos pequenos, o que reduz os custos da sua manutenção em laboratório, mas suficientemente grandes para poderem ser "manuseados" individualmente, quando necessário, sem grande esforço (Baudo 1987, Koivisto 1995). Estes crustáceos têm um desenvolvimento directo e apresentam crescimento mesmo após a maturidade, num ciclo de vida curto e muito produtivo no que diz respeito às taxas reprodutivas (Baudo 1987, Sommer & Stibor 2002, Lampert 2006). Estas características facilitam a obtenção de organismos em número suficiente nos mais diversos estádios de crescimento e permitem a realização de estudos detalhados (*e.g.*, tabelas de história de vida ou variações populacionais), satisfazendo grande parte dos desenhos experimentais. Por outro lado, *Daphnia* é

um organismo partenogenético cíclico, ou seja, no seu ciclo de vida a reprodução assexuada predominante pode alternar com a sexuada; em laboratório, sob condições controladas, é possível manter indefinidamente estes organismos sem que ocorra reprodução sexuada, o que permite o usufruto de todas as vantagens associadas ao controlo absoluto da variabilidade genotípica em contexto experimental (Koivisto 1995, Deng & Lynch 1996). As características até agora enunciadas justificam por si a utilização de *Daphnia* como organismo experimental. No entanto, é o enquadramento ecológico deste crustáceo e a sua indubitável importância nos sistemas pelágicos de água doce que favorece o seu reconhecimento como modelo experimental (Lampert 2006) em estudos de cariz ecológico e ainda em trabalhos mais específicos no âmbito da Ecotoxicologia.

Numa perspectiva algo simplificada, mas suficiente e não redutora para o enquadramento ecológico dos cladóceros no contexto do presente trabalho, pode esquematizar-se a estrutura trófica pelágica (entendida como partição da biomassa em níveis tróficos e comunidades bióticas, *sensu* Leibold et al. 1997) considerando quatro grupos funcionais fundamentais (FIGURA I.1): o fitoplâncton, o zooplâncton, o conjunto dos planctívoros vertebrados e invertebrados, e ainda o grupo funcional de topo constituído pelos organismos piscívoros.

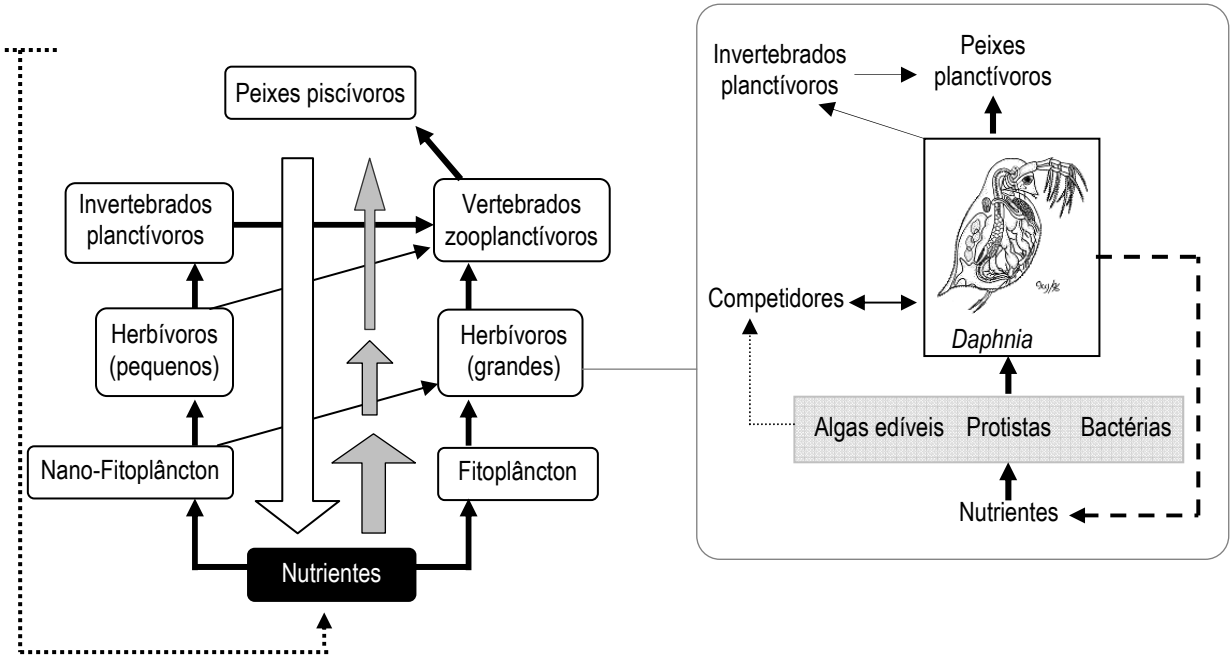


FIGURA I.1 | Representação esquemática simplificada da teia trófica da zona pelágica de um ecossistema lêntico, com referência aos mecanismos fundamentais envolvidos na regulação da estrutura trófica (controlo *top-down* [⇨]; controlo *bottom-up* [⇨]; a espessura das setas sugere a intensidade dos mecanismos). À direita, na figura, representam-se de forma mais detalhada as interacções tróficas que determinam o papel central de *Daphnia* nestes sistemas. Adaptado de Carpenter 1985, Molles 1999 e Lampert 2006; ilustração de *Daphnia* obtida no site *Biodidac: a bank of digital resources for teaching Biology* (http://biodidac.bio.uottawa.ca; acesso em Janeiro 2008).

O zooplâncton é composto por três grupos dominantes de organismos: os rotíferos, os copépodes e os cladóceros (Wetzel 1993). A FIGURA I.1. descreve os cladóceros (em geral considerados herbívoros grandes), e em especial os pertencentes ao género *Daphnia*, como organismos zooplanctónicos que desempenham um papel ecológico central na teia alimentar pelágica (Sommer & Stibor 2002, Lampert 2006), independentemente da perspectiva que se adopte para caracterizar a sua estrutura trófica (revisão por Leibold et al. 1997). Assumindo que os mecanismos de regulação dos ecossistemas funcionam obedecendo a um controlo do tipo *bottom-up*, que reflecte o efeito de factores abióticos/disponibilidade de nutrientes na produtividade em biomassa dos vários níveis tróficos, os cladóceros, no contexto da competição pelos recursos disponíveis, acabam por ser um elo fundamental na transferência de energia dos produtores para os níveis superiores da cadeia trófica (McQueen et al. 1986). Por outro lado, ao adoptar a perspectiva de que os ecossistemas são regulados por sistemas *top-down*, os cladóceros assumem-se como um item alimentar preferencial para os seus predadores vertebrados, em função das suas dimensões, e as variações nas suas populações devidas à intensidade da predação têm consequências directas na intensidade da herbívoria sobre o fitoplâncton. É também reconhecida neste contexto a relevância da reciclagem de nutrientes pelos cladóceros, como factor complementar à herbívoria, na indução de variações na composição específica e consequentemente na produtividade primária do ecossistema (Brooks & Dodson 1965, Hall et al. 1976, Carpenter et al. 1985). Está documentada na bibliografia a capacidade reguladora quer dos mecanismos via interacções *top-down*, quer via interacções *bottom-up*, sendo que a exclusão de qualquer das teorias enquanto elemento esclarecedor das variações na estrutura trófica dos ecossistemas é puramente artificial porque os dois mecanismos são, de facto, complementares na regulação do equilíbrio ecológico (Carpenter et al. 1985, Wetzel 1993, Leibold et al. 1997, Gliwicz 2001).

No âmbito destes dois mecanismos potencialmente reguladores da estrutura trófica dos ecossistemas aquáticos, as populações de *Daphnia* devem ser destacadas face aos restantes grupos zooplanctónicos e mesmo mais particularmente face aos restantes cladóceros. Foi já demonstrado, por exemplo, que a transferência de efeitos via *top-down* dos níveis tróficos superiores (peixes piscívoros e planctívoros) é mais eficaz em lagos em que a herbívoria é feita predominantemente por *Daphnia* (Pace 1984, McQueen et al. 1986, Leibold et al. 1997), o que estará relacionado quer com a capacidade superior destes organismos na supressão de fitoplâncton (Gliwicz 1990), quer com o facto de se tratarem de itens alimentares preferenciais para os

predadores visuais, dado o seu maior tamanho corporal relativo (Brooks & Dodson 1965, Hall et al. 1976). A interacção entre os mecanismos reguladores da estrutura trófica dos ecossistemas lênticos condiciona a dinâmica populacional ao nível dos diferentes níveis tróficos. As comunidades zooplanctónicas não são excepção, e esta interacção promove a sucessão dinâmica de populações, obedecendo a padrões de sazonalidade bastante bem estabelecidos; a dinâmica sazonal das populações zooplanctónicas é naturalmente balizada pelas características morfo-fisiológicas e pela flexibilidade permitida pelo ciclo de vida dos organismos (*vide* revisão por Castro 2007).

Caracterização do género *Daphnia*: posição taxonómica, morfologia e ciclo de vida

Os cladóceros são um dos grupos de organismos mais bem sucedidos nas águas doces continentais (Korovchinsky 2006). Actualmente considera-se a existência de cerca de 600 espécies de cladóceros, sendo que as primeiras descrições mais ou menos detalhadas destes organismos remontam aos finais do século XVII [*vide* revisão por Korovchinsky (1997)]. Os cladóceros constituem um grupo de pequenos crustáceos que apresenta uma distribuição geográfica actual praticamente ubíqua [*vide* revisão por Korovchinsky (2006)]; o seu registo fóssil confirma que se trata de um grupo bastante primitivo, tendo mesmo sido recentemente confirmada a sua presença, nalguns casos registando abundâncias consideráveis, em sedimentos do Paleozóico tardio e do Mesozóico (Korovchinsky 2006, Kotov & Korovchinsky 2006, Kotov 2007). Estes organismos são colocados na Classe Branchiopoda (Arthropoda: Branchiopoda), uma classe taxonómica cujos organismos integrantes se distinguem morfologicamente pela presença de apêndices torácicos em forma de folha através dos quais é feita a captação do oxigénio necessário à função respiratória (*e.g.* Ruppert & Barnes 1994).

A classificação taxonómica dos cladóceros dentro da Classe Branchiopoda não tem sido matéria consensual, muito devido à fina diferenciação morfológica contrastante com taxas elevadas de variabilidade fenotípica apresentadas pelos organismos, bem como ao facto de se tratar de um grupo que começou a ser estudado em detalhe relativamente tarde [*vide* revisão por Korovchinsky (1997)]. A última revisão tradicional feita neste contexto por Fryer (1987) reconsidera os cladóceros filogenética e taxonomicamente como um grupo artificial polifilético, que reúne 4 Ordens distintas da Classe Branchiopoda: Haplopoda, Ctenopoda, Anomopoda e Onychopoda. Mais recentemente, com o apoio de técnicas moleculares de sequenciação de fragmentos conservados do ADN mitocondrial,

assume-se que os cladóceros constituem um grupo monofilético (Schwenk et al. 1998, Taylor et al. 1999, Negrea et al. 1999, Braband et al. 2002) que reúne pelo menos 3 Ordens distintas: Ctenopoda, Anomopoda e Onychopoda (as duas primeiras são filogeneticamente mais próximas). A posição taxonómica da Ordem Haplopoda não está completamente esclarecida e o seu reconhecimento como Ordem integrante dos Cladocera não é pacífico (Negrea et al. 1999, Braband et al. 2002, Korovchinsky 2006).

Na prossecução deste trabalho foram exclusivamente utilizados cladóceros pertencentes ao Género *Daphnia*, um género bastante bem distribuído em todas as regiões climáticas, embora mais representado nas regiões temperadas (Benzie 2005), e que se integra na sistemática da Família Daphniidae, uma das Famílias da Ordem Anomopoda (Alonso 1996, Schwenk et al. 1998). Em geral, *Daphnia* caracteriza-se morfologicamente por ter a cabeça estritamente ligada com o corpo revestido com uma carapaça quitinosa com algum grau de transparência, formada por duas valvas fundidas ventralmente; possui um único olho composto na zona antero-ventral da cabeça e algumas espécies apresentam ainda um ocelo; o corpo termina com um par de garras pós-abdominais e o primeiro dos 5 apêndices torácicos apresenta dimorfismo sexual (Alonso 1996, Benzie 2005). Os ovários das fêmeas desenvolvem-se lateralmente em relação ao tubo digestivo; os ovos produzidos nos ovários são expelidos para a câmara de incubação ou marsúpio, onde, quando se trata de ovos partenogenéticos, se desenvolvem completamente (revisão da embriogénese em *Daphnia* por Kotov & Boikova 2001), originando neonatos formados que são libertados para o meio exterior (FIGURA I.2).

Embora a taxonomia de base morfológica referente ao género *Daphnia* seja fortemente condicionada pela grande plasticidade fenotípica apresentada pelas espécies e pela prevalência de híbridos inter-específicos (*e.g.*, Schwenk 1993, Schwenk & Spaak 1995), à luz de evidências moleculares relativamente recentes, é razoável considerar a sua subdivisão nos sub-géneros *Ctenodaphnia*, *Daphnia* e *Hyalodaphnia*, estes dois últimos filogeneticamente mais próximos (Schwenk et al. 2000). O trabalho experimental associado a esta dissertação foi desenvolvido utilizando uma população de *Daphnia magna*, pertencente ao subgénero *Ctenodaphnia* e três populações representantes do complexo *Daphnia longispina*, pertencente ao sub-género *Hyalodaphnia* (FIGURA I.2). *Daphnia magna* Straus, 1820, é talvez a espécie de cladóceros mais usada como organismo-modelo e mais recomendada para procedimentos padronizados no âmbito da Ecotoxicologia (*e.g.*, OECD 1998, OECD 2004). Trata-se de uma espécie de tamanho considerável, dado que a fêmea adulta pode atingir cerca de 6mm de comprimento (eixo de

medição *sensu* Pereira et al. 2004). Esta espécie é fundamentalmente eurialina, com uma distribuição geográfica holoártica e africana, preferindo massas de água de pequena dimensão e reduzida turbidez em zonas temperadas, permanentes ou mesmo temporárias, por vezes eutróficas, e tolera gamas de pH entre 6,5 e 9,9 (Alonso 1996, Benzie 2005). Não há registo da presença de populações naturais de *Daphnia magna* em Portugal. O complexo *D. longispina* é entendido actualmente (K. Schwenk, comunicação pessoal) como sendo composto pelas espécies *D. galeata* (G.O. Sars, 1854), *D. cucullata* (G.O. Sars, 1862) e *D. longispina* (O.F. Müller, 1776). Estas espécies distribuem-se na ecoregião holoártica, por vezes confinando-se à sub-região paleártica (Taylor et al. 1996), e podem ser encontradas em águas estagnadas ou com correntes leves, geralmente pouco mineralizadas. A fêmea adulta pode atingir cerca de 2.5 mm de comprimento (eixo de medição *sensu* Pereira et al. 2004), sendo que a presença de ciclomorfoses está documentada, particularmente em *D. galeata* (Alonso 1996, Taylor et al. 1996, Benzie 2005). As populações pertencentes ao complexo *Daphnia longispina* (espécies propriamente ditas e híbridos inter-específicos) estão bem representadas em lagos e albufeiras distribuídos por todo o território de Portugal continental (BB Castro & JL Pereira, observações não publicadas; Caramujo & Boavida 2000, Geraldes & Boavida 2004, Abrantes et al. 2006, Castro 2007).

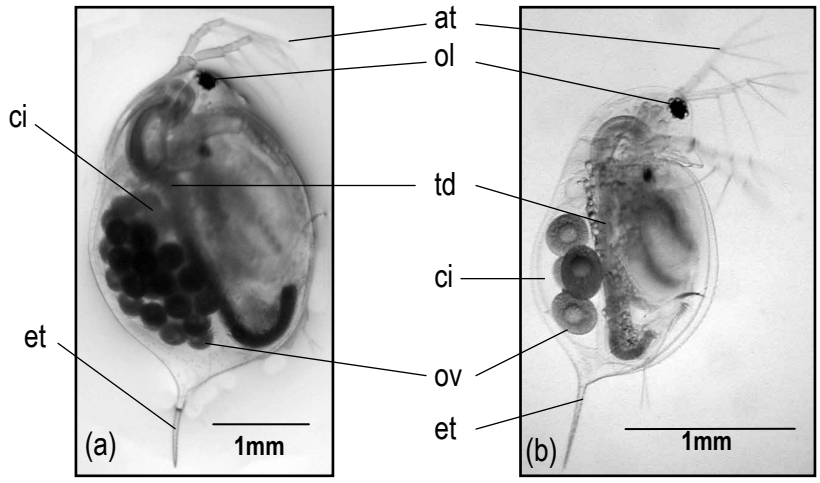


FIGURA I.2 | Fotografias originais de fêmeas adultas de (a) *Daphnia magna* e *Daphnia* cf *longispina* (b) carregando ovos na câmara de incubação, com breve referência à sua morfologia geral: (at) antenas (locomoção/natação); (ol) olho; (ci) câmara de incubação; (ov) ovos partenogenéticos; (td) tracto digestivo; (et) espinho terminal.

Não obstante a inexistência de registos da ocorrência de hibridização inter-específica em *D. magna*, este é um fenómeno bastante documentado entre espécies do complexo *D. longispina*; apesar da ocorrência de hibridização, as espécies do complexo *D. longispina* continuam a considerar-se geneticamente isoladas (Schwenk 1993, Keller et al. 2007). A ocorrência de híbridos e a sua co-ocorrência com espécies parentais em lagos e reservatórios naturais têm sido objecto de estudo detalhado nos últimos anos (*e.g.*, Schwenk & Spaak 1995, Spaak 1996, Schwenk et al. 1998, Gieβler et al. 1999, Schwenk et al. 2000, Keller & Spaak 2004, Keller et al. 2007). Por natureza, os híbridos possuem uma combinação de genes originários de dois *pools* genéticos (espécies parentais) distintos. Esta condição pode gerar variações nas combinações genéticas significativamente maiores do que as resultantes da normal recombinação genética intra-específica e pode resultar em genótipos competitivamente superiores aos parentais (Schwenk & Spaak 1995), capazes de uma melhor resposta adaptativa às forças selectivas bióticas e abióticas inerentes a cada local (*Local adaptation*; De Meester 1996). A superioridade temporária dos genótipos híbridos em resposta a diversos estímulos ambientais, quando avaliada considerando isoladamente determinados parâmetros do ciclo de vida (*e.g.*, reprodução ou crescimento) ou analisando o sucesso ecológico (*fitness*) geral das populações, tem sido demonstrada no complexo *D. longispina* (*e.g.*, Boersma & Vijverberg 1994, Spaak and Hoekstra 1997, Declerck & De Meester 2003).

Tipicamente, *Daphnia* reproduz-se por partenogénese cíclica (FIGURA I.3), um processo que lhe confere vantagens adaptativas a curto e a longo prazo e permite uma maior flexibilidade na rapidez com que ultrapassa pressões evolutivas (Lynch & Gabriel 1983, Taylor et al. 1999). Este processo dita que os organismos sejam capazes de investir a curto prazo no crescimento rápido das populações, reproduzindo-se assexuadamente; paralelamente, confere-lhes a possibilidade de "optarem" pela reprodução sexuada, quando as condições ambientais se tornam desfavoráveis para o crescimento da população, gerando a diversidade genética necessária ao seu sucesso a longo prazo. Assim, numa fase ambientalmente favorável, as fêmeas partenogenéticas maduras produzirão ovos diplóides que se desenvolverão para novas fêmeas geneticamente idênticas à progenitora. O crescimento das fêmeas ocorre através da ecdise sucessiva do exoesqueleto cuticular, cuja frequência é fundamentalmente dependente das condições de temperatura e de recursos alimentares disponíveis (Threlkeld 1987). Após a maturação do sistema reprodutor (que se completa ao fim de 4-7 estádios de desenvolvimento; Threlkeld 1987), a deposição de ovos na câmara de incubação ocorre ciclicamente ao longo da vida da fêmea, em intervalos regulares de cerca de 3 dias; esses ovos desenvolvem-se completamente e os neonatos resultantes são

libertados para o meio exterior no início do processo de ecdise (Zaffagnini 1987). *Daphnia* pode prologar este ciclo reprodutivo iteropárico por bastante tempo – poderá produzir até cerca de 20 ninhadas –, sendo que, como em condições naturais a longevidade destes organismos encurta drasticamente, não deverá haver condições para que o processo reprodutivo se estenda por tanto tempo (Threlkeld 1987).

Após vários ciclos deste processo assexuado e sob um determinado estímulo ambiental, o desenvolvimento dos embriões a partir dos ovos diplóides é condicionado e as fêmeas produzem, partenogeneticamente, ninhadas de machos ou ninhadas mistas de machos e fêmeas geralmente sexuadas (*e.g.*, Hobæk & Larsson 1990, Innes 1997, Innes & Singleton 2000). Actualmente, aceita-se que a determinação sexual em *Daphnia* é fundamentalmente ambiental [*Environmental Sex Determination*, Korpelainen (1990)], no entanto, a extensão da capacidade para investir em reprodução sexuada deverá ser determinada geneticamente (Yampolsky 1992, Spaak 1995, Deng 1996, Innes & Sengleton 2000). São vários os estímulos ambientais que, por si só ou em interacção, não só podem induzir alterações em vários parâmetros do ciclo reprodutivo assexuado (*e.g.*, tempo até à maturação, capacidade reprodutiva, longevidade), como são potenciadores da reprodução sexuada em *Daphnia*. A temperatura e o fotoperíodo, por serem factores físicos indicadores de condições climáticas mais adversas para as populações, bem como a densidade populacional, que condiciona a disponibilidade de alimento promovendo o investimento nas estratégias de competição inter-específica, são exemplos típicos desses estímulos ambientais (*e.g.*, Korpelainen 1986, Hobæk & Larsson 1990, Kleiven et al. 1992, Stibor & Lampert 2000, Gyllström & Hansson 2004). A pressão predatória constitui um estímulo também capaz de afectar não só o ciclo reprodutivo assexuado (e.g. Hülsmann et al. 2004, Castro et al. 2007), mas também a taxa de indução da reprodução sexuada (Lass & Spaak 2003). Já foi também demonstrada a importância dos efeitos maternais (De Meester et al. 1998, Boersma et al. 2000) e da exposição a químicos sintéticos exógenos de diversas naturezas (Olmstead & LeBlanc 2000, Zhang & Baer 2000, Kashian & Dodson 2004) na indução da reprodução sexuada em *Daphnia*.

No culminar do processo de reprodução sexuada, as fêmeas produzem ovos haplóides (logo após a produção de machos; Hebert 1987) com uma constituição bioquímica distinta das dos ovos diplóides, de modo a assegurar a sua condição durante toda fase de dormência/resistência que possam vir a sofrer até à germinação (Pauwels et al. 2007). Depois de fecundados pelos machos, num processo que se assume ser aleatório em populações naturais, os ovos são

envolvidos num conjunto de membranas protectoras designado globalmente de *ephipium*, formando estruturas de resistência (Hebert 1987) (FIGURA I.3). Quando libertadas pelas fêmeas, estas *ephippia* tanto podem flutuar à superfície da água, como depositar-se no sedimento ou mesmo sofrer, de imediato ou posteriormente, processos vários de dispersão (Havel & Shurin 2004). Sob condições ambientais favoráveis, que no caso dos cladóceros geralmente coincidem anualmente com o início da Primavera, as *ephippia* eclodem, originando sempre fêmeas que iniciam sempre o ciclo assexuado (Hebert 1987, Brendonck & De Meester 2003).

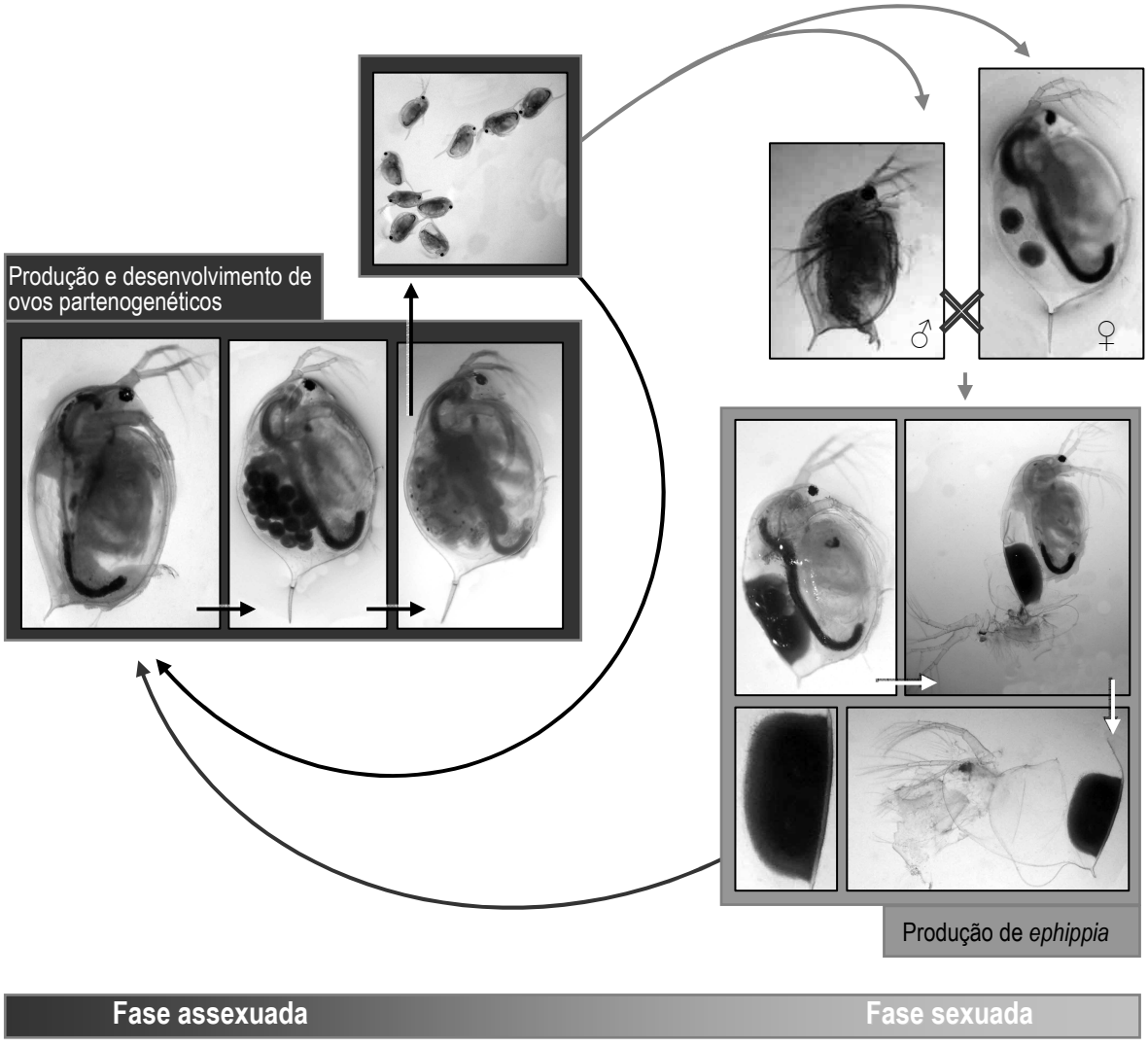


FIGURA I.3. | Representação esquemática da reprodução partenogenética cíclica de *Daphnia* (*Daphnia magna*). Todas as fotografias incluídas na figura são originais, excepto a que evidencia o macho de *Daphnia magna* que foi adaptada da versão Web de Olmstead & LeBlanc 2007 (http://www.biolsci.org/v03p0077.htm; acesso em Janeiro 2008).

Com o processo de eclosão das *ephippia*, ocorre a renovação da estrutura genética da população e a exposição dos novos genótipos às condições ambientais, bem como a todo o processo de selecção clonal inerente. As forças selectivas actuarão sobre os novos clones, erodindo a variabilidade genética expressa até um nível óptimo, passível de ser sustentado exclusivamente com o processo reprodutivo partenogenético; ao mesmo tempo, e dependendo das directrizes da pressão selectiva (condições ambientais), pode ocorrer o restabelecimento de alguma variabilidade genética não expressa em gerações anteriores (*hidden genetic variance*, *sensu* Lynch 1985; Brendonck & De Meester 2003). Em suma, a estrutura genética das populações de *Daphnia* é fundamentalmente determinada pelo equilíbrio entre a selecção clonal, com a consequente erosão genética característica da fase partenogenética, e o restabelecimento da variabilidade genética pelos clones provenientes dos bancos de *ephippia* (DeMeester 1996, De Meester et al. 2006). Esta dinâmica genética associada ao processo de partenogénese cíclica condiciona toda a dinâmica de evolução fenotípica das populações de *Daphnia* (Lynch & Gabriel 1983, Lynch 1985): a plasticidade fenotípica das populações de *Daphnia* em resposta a diversos estímulos ambientais, determinante na definição da tolerância a esses mesmos estímulos, é sempre limitada pela variabilidade de genótipos existentes (Lynch & Gabriel 1987).

Disponibilidade de alimento como estímulo condicionador do *fitness* de *Daphnia*

O fitoplâncton constitui o principal suporte alimentar natural de *Daphnia*, muito embora os cladóceros sejam considerados filtradores não-selectivos, no sentido lato do termo: itens como bactérias, protozoários ciliados e flagelados, detritos e outras partículas sólidas suspensas variadas podem completar o espectro alimentar destes organismos (Lampert 1987), desde que cumpram uma gama de tamanhos consistente com as dimensões da malha do seu aparelho filtrador, isto é, dentro da gama <10µm-150 µm (DeMott 1982, Lair 1991). A composição qualitativa do espectro alimentar (quer em termos de composição, quer de qualidade mineral e bioquímica) pode influenciar decisivamente a condição dos organismos, o seu crescimento, a reprodução e, em última instância, o *fitness* das populações e a sua composição específica. Por exemplo, foi já associada a presença de cianobactérias filamentosas na dieta alimentar com a exclusão de espécies de maior tamanho corporal em comunidades de *Daphnia* (DeMott et al. 2001), ou com decréscimos efectivos no crescimento e reprodução de *Daphnia* (Repka 1997). Estes fenómenos estarão relacionados com a baixa digestibilidade das cianobactérias (Vanni & Lampert 1992), com a interferência de base

morfológica com os mecanismos de filtração de *Daphnia*, com o seu deficiente conteúdo nutricional (Repka 1997, Repka 1998, DeMott et al. 2001, Ghadouani et al. 2007), ou eventualmente com a síntese de cianotoxinas (Trubetskova & Haney 2006). A composição mineral – rácio Carbono: Fósforo/deficiência de Fósforo - e as limitações bioquímicas em ácidos gordos essenciais na dieta alimentar têm demonstrado ser também um factor decisivo para o crescimento, reprodução e crescimento populacional de *Daphnia* (Plath & Boersma 2001, Becker & Boersma 2003, Becker & Boersma 2005). Rellstab & Spaak (2007) demonstraram ainda que variações na concentração de partículas sólidas suspensas no seston podem promover alterações, quer nos parâmetros reprodutivos e de crescimento de *Daphnia hyalina*, quer no seu *fitness* enquanto população natural.

Para além da variação qualitativa na sua composição, as comunidades fitoplanctónicas naturais sofrem flutuações quantitativas sazonais associadas às alterações naturais das condições bióticas e abióticas (Sommer et al. 1986). Estas flutuações na disponibilidade de recursos alimentares para o zooplânkton herbívoro constituem uma variável sazonal que, de uma forma muito directa, condiciona a história de vida dos zooplanctontes, influenciando decisivamente a sua dinâmica populacional. Em geral, a resposta mais imediata de *Daphnia* à diminuição quantitativa da disponibilidade de recursos, em última instância entendida enquanto limitação da quantidade de Carbono disponível para todas as funções vitais, envolve ajustes no crescimento somático e na capacidade reprodutiva individual. Quando a quantidade de alimento ingerido diminui, observa-se uma diminuição significativa no crescimento somático de *Daphnia* (Tessier & Goulden 1987, Lynch 1989, Lynch 1992); este padrão reflecte os custos energéticos associados com o processo de ecdise, que aumentam proporcionalmente com o aumento do tamanho do organismo, e não está relacionado com variações no número de estádios de desenvolvimento que se sucedem ao longo da vida de *Daphnia* (Lynch 1989, Lynch 1992). Por outro lado, tendo que lidar com baixos níveis de Carbono, estes organismos precisam de mais tempo para atingir o tamanho corporal mínimo necessário para iniciar a reprodução; logo, ocorrerá frequentemente um atraso significativo da primeira reprodução (Lynch 1989, McCauley et al. 1990a). Naturalmente que a fecundidade, isto é, a quantidade de ovos e/ou de descendência viável, também registará valores mais baixos à medida que a quantidade de alimento disponível diminui (*e.g.*, Lampert 1978, Guisande & Gliwicz 1992, Trubetskova & Lampert 1995), podendo mesmo ser nula sob escassez alimentar extrema (*e.g.*, Guisande & Gliwicz 1992, Trubetskova & Lampert 1995).

As consequências da limitação da disponibilidade de Carbono na qualidade dos ovos produzidos (ou seja na sua provisão com reservas lipídicas maternas) e, consequentemente, no desenvolvimento e probabilidade de sobrevivência da descendência, não são consensuais. Lynch (1989) observou uma diminuição no volume dos ovos produzidos consistente com a diminuição do nível alimentar e um maior tamanho dos neonatos em concentrações de Carbono não limitantes. No entanto, outros estudos demonstraram uma tendência para o aumento do tamanho e/ou da qualidade dos ovos produzidos por fêmeas sujeitas a rações alimentares mais limitadas, relativamente aos produzidos em tratamentos com recursos ilimitados (Guisande & Gliwicz 1992). Os argumentos a favor desta última tese consideram que, até um determinado valor crítico – a partir do qual simplesmente não há energia suficiente para ocorrer produção de propágulos –, a resposta de *Daphnia* à redução alimentar consistirá na produção de menos ovos, sendo que os que forem produzidos serão maiores e de qualidade superior, de forma a aumentar a probabilidade de sobrevivência dos neonatos se as condições alimentares entretanto não se tornarem mais favoráveis (Guisande & Gliwicz 1992). O tamanho dos ovos e o tamanho dos neonatos são parâmetros positivamente correlacionados (Glazier 1992) e neonatos maiores, portanto resultantes de ovos maiores, sobreviverão melhor em condições de privação alimentar (Gliwicz & Guisande 1992); por outro lado, o seu período pré-reprodutivo será mais curto (Ebert 1991) e, consequentemente, a probabilidade conseguirem atingir o tamanho crítico a partir do qual podem iniciar a reprodução aumenta. Quando as condições alimentares não são limitantes, a tendência passa a envolver a produção de muitos ovos, mas mais pequenos, sendo que os neonatos deles resultantes não têm a capacidade de sobreviver muito tempo em condições de privação alimentar (Gliwicz & Guisande 1992).

Glazier (1992) sintetizou estas observações, e considerando uma abordagem mais abrangente das respostas de *Daphnia* a gradientes de concentração alimentar, propôs um modelo detalhado para explicar a relação entre a disponibilidade de recursos e as estratégias reprodutivas: (i) em níveis alimentares muito baixos, o tamanho dos neonatos variará numa razão directamente proporcional com a variação da quantidade de alimento, ou seja, os ovos são produzidos e enriquecidos à medida que os recursos alimentares proporcionam a energia necessária para o efeito (*Reproductive constraints*); (ii) em níveis alimentares médios, nos quais a restrição energética não é extrema e, consequentemente, é possível haver alguma gestão do investimento na reprodução, a relação entre a concentração de alimento e o tamanho dos neonatos é inversamente proporcional (*Adaptative response*); (iii) numa gama de concentrações de alimento não limitante,

não há variações no tamanho dos neonatos produzidos que possam ser directamente relacionadas com as variações na quantidade de alimento, uma vez que o único limite a considerar no processo reprodutivo é o tamanho mínimo que um neonato precisa de ter para ser viável. Trubetskova & Lampert (1995) obtiveram dados experimentais consistentes com o modelo descrito por Glazier. No entanto, e considerando que a relação entre o número e o tamanho dos neonatos pode depender não só das condições ambientais (*e.g.*, disponibilidade alimentar), mas também de outras variáveis como a condição materna ou o genótipo (Ebert 1993), os dados experimentais que aparentemente contradizem os pressupostos teóricos assumidos por Glazier (*e.g.*, Tessier & Consolatti 1991, Boersma 1995, Boersma 1997) não devem ser desvalorizados.

Os dafnídeos são geralmente considerados estrategas r (*r selected*), o que significa que, independentemente das condições ambientais, estes organismos deverão privilegiar a produção de descendência (Gabriel 1982). De facto, há evidências experimentais que confirmam que, em condições óptimas, uma grande parte da energia disponível é gasta na reprodução, sendo a restante investida na respiração celular e em pequena escala no crescimento (Taylor & Gabriel 1992). No entanto, numa abordagem mais realista, considera-se que estes organismos gerem a energia disponível sob um compromisso entre as estratégias que maximizam o crescimento populacional e as estratégias que maximizam a produção de descendência (Taylor & Gabriel 1985). A gestão destas estratégias, em condições flutuantes de disponibilidade alimentar, traduz-se, fisiologicamente, na regulação da alocação da energia disponível para as várias funções vitais e, "macroscopicamente", no equilíbrio entre o crescimento somático dos organismos e a sua capacidade reprodutiva.

A conceptualização da alocação de energia em *Daphnia* foi desenvolvida de uma forma integradora por Kooijman (1986) e McCauley et al. (1990b). Ambos os modelos privilegiam a reprodução em relação às restantes funções, assumindo que uma percentagem fixa da energia disponível é alocada para essa função e só o restante é distribuído pelo crescimento e manutenção. A diferença fundamental entre os dois modelos reside na posição que assume o armazenamento de energia (*somatic storage*) relativamente aos fluxos energéticos. Assim, Kooijman (1986) assume que a energia assimilada é directamente armazenada, e só depois é feita a alocação para o crescimento estrutural, para a manutenção fisiológica e para a reprodução; McCauley (1990b) defende que a energia armazenada só é utilizada para processos de manutenção fisiológica e a que vai sendo adquirida é imediatamente alocada para os processos de produção estrutural e

reprodutiva. Em condições de privação alimentar, ou seja, de limitação energética, o modelo de Kooijman prevê a alocação prioritária das reservas para a manutenção fisiológica, sendo depois feito o investimento energético na reprodução e no crescimento, por esta ordem. O modelo de McCauley e colaboradores prevê que, à medida que ocorre depleção de recursos, o crescimento e a reprodução abrandarão ou cessarão, seguindo-se um período de exploração das reservas energéticas exclusivamente direccionada para a manutenção fisiológica. Em Gurney et al. (1990) é reforçado o modelo de McCauley et al. (1990b) e são sugeridas as adaptações que podem ser feitas ao modelo em condições alimentares flutuantes, com base em evidências experimentais reportadas na literatura. A recuperação fisiológica de *Daphnia* quando as condições alimentares se tornam mais favoráveis foi revista experimentalmente no ano seguinte por Bradley et al. (1991) e mais tarde por Polishchuk & Vijverberg (2005). Naturalmente que as estratégias de alocação de energia adoptadas por *Daphnia* em diferentes regimes alimentares têm sido exploradas em detalhe desde a publicação dos modelos acima referidos, e é considerável a reunião de evidências experimentais, mais específicas no mecanismo analisado, geradas sobre a generalização teórica exposta nesses modelos (e.g. Taylor & Gabriel 1992, Taylor & Gabriel 1993, Glazier 1998, Rinke & Vijverberg 2005).

A importância da disponibilidade de recursos alimentares não se extingue na análise dos seus efeitos fisiológicos directos sobre os indivíduos que deles dependem. A variação dos recursos alimentares e a sua influência sobre um determinado organismo são processos que devem ser entendidos como mecanismos integrados numa rede de interacções ecológicas complexa. A competição inter-específica, e em alguns casos intra-específica, é um dos factores ecológicos que melhor reflecte a forma como as populações naturais utilizam os recursos alimentares disponíveis. Num cenário de limitação alimentar, a coexistência ou a extinção de espécies competidoras pode ser regulada pela capacidade que essas mesmas espécies demonstram na utilização dos recursos (Tilman 1980, revisto em Miller et al. 2005). No contexto específico das populações naturais de *Daphnia* – e dado o seu papel ecológico central na estrutura trófica pelágica lacustre –, esta questão tem sido abordada fundamentalmente através da exploração da *Size-Efficiency Hypothesis* (SEH) (Brooks & Dodson 1965), que explora a influência do tamanho corporal das espécies nas interacções inter-específicas de competição e predação (Brooks & Dodson 1965, Hall et al. 1976). Em geral, e no que diz respeito exclusivamente à capacidade de exploração de recursos alimentares, a SEH considera que os herbívoros zooplanctónicos com maior tamanho corporal serão competitivamente superiores aos de menor tamanho, uma vez que são capazes de uma

maior eficácia no mecanismo de filtração de alimento e de uma maior eficácia metabólica (o seu metabolismo requer menos energia por unidade de massa corporal).

Gliwicz (1990) verificou experimentalmente a SEH utilizando várias espécies de *Daphnia* e *Ceriodaphnia reticulata*, demonstrando que, em condições de restrição de recursos alimentares, as espécies de maior tamanho corporal são de facto competitivamente superiores às mais pequenas. Vários outros autores obtiveram resultados consistentes com a exclusão competitiva das espécies de menor tamanho corporal, em condições de restrição alimentar e ausência de predação (*e.g.*, Lynch 1992, Kreutzer & Lampert 1999). No entanto, a dinâmica das populações de *Daphnia* sob o enquadramento teórico da SEH não é consensual, tendo sido encontradas evidências experimentais que não são consistentes com os seus postulados teóricos (*e.g.*, Lynch 1977, DeMott 1982, Tillmann & Lampert 1984, Tessier & Goulden 1987). Assim, vários outros factores têm sido apontados como reguladores da competição inter-específicas e capazes, portanto, de minimizar o efeito do tamanho corporal no sucesso competitivo das espécies de cladóceros. Por exemplo, a estrutura intra-populacional pode ser bastante relevante, dado que os requisitos metabólicos e as estratégias energéticas de uma população predominantemente constituída por adultos diferem largamente dos de uma população em que a cohorte juvenil é dominante (Lynch 1977, Tessier & Goulden 1987). O grau de plasticidade fenotípica associada a cada genótipo pode também influenciar decisivamente a capacidade das populações de *Daphnia* na exploração dos recursos alimentares (Epp 1996) e a eficiência de filtração, bem como os requisitos metabólicos e a gestão da energia disponível não deverão ser função exclusiva do tamanho corporal específico (Lynch 1977, DeMott 1982).

Independentemente de se centrar mais em processos fisiológicos individuais ou de contemplar adicionalmente interacções ecológicas associadas, esta reflexão sobre a exploração de recursos alimentares conduz ao reconhecimento de que o alimento acaba por ser um estímulo condicionador do *fitness* das populações naturais de *Daphnia* e, em última instância, da evolução específica e/ou clonal dessas mesmas populações. Em qualquer comunidade de cladóceros, a flutuação sazonal dos recursos alimentares não só influencia a produtividade dos genótipos existentes ao interferir com a fisiologia e a energética do seu ciclo de vida, como acaba mesmo por condicionar a selecção dos genótipos que melhor conseguem explorar os padrões associados à sazonalidade fitoplanctónica.

Pesticidas: xenobióticos com potencial para afectar o ecossistema aquático

De acordo com a União Europeia (CE 1991), define-se como pesticida – produto fitofarmacêutico – qualquer substância activa ou preparação que se destine a prevenir, destruir, repelir ou mitigar organismos potencialmente prejudiciais para os vegetais beneficiados, assegurando portanto a sua conservação. Os pesticidas são, assim, agentes químicos desenhados com a intenção fundamental de inibir a actividade biológica de organismos prejudiciais à produção agrícola, sejam eles plantas (herbicidas), insectos (insecticidas), fungos (fungicidas) ou outros. Os benefícios económicos dos pesticidas estão bem expressos na sua definição de base, reflectindo-se principalmente no facto de serem agentes primordiais no aumento, ou pelo menos na manutenção, dos níveis de produção agrícola e na redução dos custos associados. É importante atribuir também aos pesticidas alguns benefícios ecológicos, que se definem por comparação com outros métodos de controlo de pragas, tais como o cultivo excessivo e contínuo, que, para fazer face aos prejuízos induzidos por uma determinada praga, pode aumentar drasticamente a erosão do solo, tornando-o impraticável como solo agrícola. Há que referir ainda os benefícios sociais dos pesticidas já que estes não só contribuem para a produção de géneros alimentares a baixo custo, como também reduzem o risco de perda de culturas e podem até ajudar a controlar vectores de algumas doenças epidémicas (Russell 1995).

A necessidade de um aumento quantitativo e qualitativo da produção agrícola estimulou o aumento significativo da utilização de pesticidas e outros produtos de protecção de culturas, ou seja, de produtos fitofarmacêuticos. Confinando os dados à realidade da União Europeia (UE), foi registado um franco aumento das vendas de pesticidas (fungicidas, herbicidas e insecticidas) até ao fim dos anos 90, sendo que desde aí tem sido observado um ligeiro declínio, que é atribuído fundamentalmente ao esforço dos países constituintes da Europa dos 15 (EC 2007). Apesar desta ligeira queda dos índices de venda nos últimos anos, é facto que em 1995 se vendiam na UE 279811 toneladas de ingredientes activos constituintes de pesticidas, sendo que em 2002 esse valor ascendeu para 327642 toneladas (EUROSTAT: http://epp.eurostat.ec.europa.eu; acesso em Janeiro de 2008). A realidade nacional segue as mesmas tendências, ou seja, embora seja assumido um progressivo declínio nas vendas nos últimos anos, não há ainda sinais indubitáveis de retorno aos índices da década passada: em 1995 vendiam-se 11818 toneladas enquanto em 2005 se venderam 16346 toneladas de princípios activos (EUROSTAT: http://epp.eurostat.ec.europa.eu; acesso em Janeiro de 2008). O decréscimo nas vendas de pesticidas é atribuído sobretudo ao desenvolvimento de novos

compostos, desenhados de forma a diminuir a dosagem necessária, e não à efectiva diminuição da utilização de produtos fitofarmacêuticos para a protecção de culturas agrícolas (EC 2007).

A UE reconhece a necessidade de utilização de produtos fitofarmacêuticos para assegurar elevados níveis (em quantidade e qualidade) de produtividade agrícola (CE 2005). A protecção da saúde humana relativamente à utilização destes produtos é assegurada legalmente nos países comunitários através do Regulamento nº 396/2005 (CE 2005) que estabelece os limites máximos de pesticidas no interior e à superfície dos géneros alimentícios, actualizando e consolidando legislação sucessiva sobre a matéria publicada desde 1976 (CE 2005). Por outro lado, a legislação europeia actual – também resultante da revisão e actualização de legislação anterior – relativa à água para consumo humano (CE 1998) estabelece limites máximos para a presença de pesticidas na água fornecida pelas redes de distribuição (0,1µg/L por pesticida e 0,5µg/L para o conjunto dos pesticidas detectados). A preocupação paralela com a protecção ambiental subsequente à utilização de pesticidas é um processo bastante mais recente no contexto da UE, remontando apenas a 1991, ano em que é publicada a Directiva 91/414/CEE (CE 1991). Esta directiva regula a colocação de produtos fitofarmacêuticos no mercado, exigindo análises de risco extensivas, quer aplicadas à população humana, quer ao ambiente, como condição fundamental para a obtenção da autorização para o seu comércio livre no espaço da UE. A Directiva constituiu o ponto de partida para a publicação de variadíssimos documentos a ela associados e referentes às recomendações instituídas. É com a publicação de, entre outros, documentos com linhas orientadoras relativas à ecotoxicologia terrestre (EC 2002a) e à ecotoxicologia aquática (EC 2002b), que é plenamente reconhecida a importância da protecção destes ecossistemas no contexto comunitário, como mecanismo inerente à regulamentação da comercialização e uso de pesticidas para melhorar a produção agrícola. Mais recentemente, é publicado um documento que regula o registo, avaliação, autorização e restrição de substâncias químicas (REACH) no contexto europeu (CE 2006). Embora remetendo os pormenores mais técnicos relativos aos produtos fitofarmacêuticos para a Directiva 91/414/CEE (CE 1991) e documentos dela derivados, o Regulamento REACH reconhece a urgência de recolher e compilar informação ecotoxicológica acerca destes produtos, quer no contexto ambiental, quer no da saúde humana.

O pesticida perfeito seria aquele que desaparecesse pouco depois da sua aplicação nos locais-alvo e da eliminação ou inactivação da peste-alvo, sem quaisquer perdas laterais e/ou efeitos colaterais (Cohen et al. 1995). Tal químico não existe e os pesticidas, enquanto fonte de poluição

difusa, podem mesmo ser considerados uma das maiores ameaças de contaminação de recursos hídricos superficiais ou subterrâneos (Loague et al. 1998). O compartimento aquático é integrador dos vários processos biogeoquímicos em regiões agrícolas, logo, será o destino final dos resíduos dos pesticidas aplicados, seja ao nível das massas de água superficiais (rios, lagos, lagoas ou charcos temporários que se distribuam na zona da produção ou mesmo em áreas periféricas) ou ao nível das massas de água subterrâneas. São vários os processos físicos que fomentam esta interacção com potencial para gerar efeitos adversos a diferentes níveis do ecossistema aquático. Estes processos são dependentes da natureza (ou seja, das propriedades físicas e químicas) da(s) substância(s) activa(s) constituinte(s) dos pesticidas, das condições agro-climáticas e ainda do sistema de aplicação utilizado; é ainda reconhecido que as más condições de utilização, a ocorrência de acidentes durante a aplicação e o uso ilegal de pesticidas constituem vias adicionais de entrada significativa destes compostos no sistema aquático (Carter 2000).

Nas revisões mais actuais, relativas à contaminação do compartimento aquático por pesticidas e seus resíduos, os autores referem-se a cinco vias fundamentais de poluição através das quais o processo pode ser facilitado (Brown et al. 1995, Flury 1996, Carter 2000, Huber et al. 2000, Reichenberger et al. 2007): deriva resultante de aspersão (*spray drift*), volatilização (*volatilisation and precipitation*), escorrência (*surface runoff*), lixiviação (*leaching*) e drenagem (*drainflow and throughflow*). Quando um pesticida é aplicado por aspersão é relativamente comum a deriva directa de resíduos e a sua consequente deposição no sistema aquático, sendo que a diluição e dissipação do produto, bem como a sua biodisponibilidade para organismos aquáticos não-alvo, são condicionadas pela formulação do produto, pelas características da substância activa, pelas condições climáticas, pelo tipo de cultura e ainda pelas características da própria massa de água (velocidade da corrente, a presença de vegetação e o tipo de sedimento). A volatilização de pesticidas aplicados pode ser considerada uma via comparativamente residual; embora as perdas de pesticidas voláteis após a aplicação possa atingir valores consideráveis, estes não são tipicamente detectados em sistemas aquáticos e os seus resíduos são detectáveis apenas em quantidades residuais na água da chuva. A escorrência à superfície parece ser uma via primordial de entrada de pesticidas nas águas superficiais, sendo que a sua importância aumenta quando os pesticidas são aplicados sob elevada pluviosidade e o solo excede a sua capacidade de absorção e infiltração, e/ou quando há declives acentuados no terreno de cultivo. A lixiviação é um processo relevante de transporte de resíduos directamente para águas subterrâneas, dependente em larga escala das características, textura e estrutura do solo agrícola, do tipo de irrigação e das condições

climáticas. Estas características condicionam ainda o transporte lateral à superfície de resíduos suspensos no lixiviado directamente para águas superficiais, e por isso se considera que a lixiviação e a escorrência superficial são processos mutuamente dependentes. Por fim, deve ser destacado o transporte "oportunista" de resíduos por drenagem. As produções agrícolas são sustentados por redes de drenagem, que permitem remover a água em excesso do solo, constituindo, por vezes, canais preferenciais para a entrada de resíduos dos pesticidas no compartimento aquático. Este tipo de transporte parece ser menos relevante que o feito por escorrência superficial e não está provado que seja dependente de características intrínsecas dos pesticidas tais como a sua formulação.

Em condições naturais, e apesar de alguns estudos de cariz exclusivamente laboratorial poderem ser contraditórios, pode ser significativa a percentagem de pesticidas que se perde para o sistema e se move através dele, não actuando efectivamente na espécie-alvo (Flury et al. 1995, Flury 1996, Ribeiro et al. 2007). A mobilidade de um pesticida no solo é condicionada pela interacção de quatro factores principais (Russell 1995): (1) as propriedades moleculares do próprio pesticida como a sua solubilidade, taxas de hidrólise, pressão de vapor, coeficiente de adsorção normalizado relativamente ao teor de carbono orgânico do solo (Koc) e coeficiente de partição octanol-água (Kow)[1]; (2) as características do solo, tais como a textura e estrutura, o pH, o teor em matéria orgânica, a capacidade de retenção de água e a sua comunidade biótica; (3) as condições agro-climáticas, fundamentalmente relacionadas com a intensidade e extensão da precipitação, com padrão de temperatura e exposição solar, bem como com a topografia do terreno e o tipo de redes de drenagem; (4) o padrão de utilização, isto é, a taxa, a época do ano e o método de aplicação do pesticida. Dado o grande número de condições em jogo, só a integração de resultados obtidos em laboratório, em ensaios de campo e através da aplicação de modelos teóricos permitirá uma avaliação credível da capacidade de um pesticida para se mover através de um solo, quer horizontalmente à sua superfície, quer verticalmente ao longo do seu perfil (Russell 1995). Reconhece-se, por exemplo, que a mobilidade de determinados pesticidas no solo pode estar sub-valorizada já que, dados os custos associados à experimentação em campo, é frequentemente estimada através de representações artificiais físicas, conceptuais ou matemáticas, integradoras

[1] **Kow:** medida da distribuição da substância entre a fase lipofílica e a fase aquosa do sistema, que é indicadora da mobilidade de um determinado composto químico no solo. Valores de Kow abaixo de 20 indicam tendência para elevada mobilidade no solo, enquanto valores acima de 10^4 denunciam que se trata de um químico praticamente imóvel no solo (Russel 1995). Muito embora de uma forma pouco consensual, este parâmetro é utilizado na predição da toxicidade e potencial de bioacumulação de potenciais contaminantes (Renner 2002).

dos parâmetros reguladores da mobilidade das moléculas de pesticidas através do solo (Cohen et al. 1995, Ribeiro et al. 2007).

É notória a crescente preocupação relativa aos riscos associados ao uso de pesticidas, ao controlo e melhoramento das práticas agrícolas e às técnicas de mitigação de efeitos da poluição difusa por agro-químicos nos recursos hídricos, demonstrada tanto ao nível da comunidade científica (*e.g.*, Brock et al. 2000a,b, Caruso 2001, D'Arcy & Frost 2001, Castro et al. 2005, Chelme-Ayala et al. 2005, Guest et al. 2006, Oquist et al. 2007, Reichenberger et al. 2007), como ao nível da tomada de decisões e regulamentação (*e.g.*, Tooby 1995, Loague et al. 1998, Craven 2002). Um pouco neste contexto, e acompanhando a erradicação do mercado de alguns pesticidas muito persistentes a partir da década de 70 [*e.g.*, organoclorados, PAH's (*Polyaromatic Hydrocarbons*) ou PCB's (*Polychlorinated Biphenyls*)], surge o desenvolvimento, produção e adopção crescente de pesticidas ditos "contemporâneos", de "uso corrente", ou "benignos" (Barr & Needham 2002). Estes pesticidas tendem a não persistir muito tempo no ambiente, alguns decompondo-se em poucas semanas quando expostos à luz do sol ou a uma matriz aquática. Geralmente são selectivos quanto ao modo de acção, não bioacumulam e devem ser passíveis de ser metabolizados e excretados pelos diversos organismos não-alvo eventualmente expostos (Hutson & Roberts 1985, Barr & Needham 2002). Os pesticidas deste tipo têm mecanismos de acção variados e bastante específicos em relação ao alvo, podendo ter uma estrutura química bastante variável: organofosforados, carbamatos, triazinas, piretróides e acetanilidas são exemplos de classes químicas a que pertencem alguns destes novos pesticidas.

Não obstante, são relativamente abundantes os estudos recentes que demonstram que, no compartimento aquático, ainda é significativa a presença de resíduos de pesticidas persistentes (*e.g.,* Golfinopoulos et al. 2003, Chelme-Ayala et al. 2005, Guest et al. 2006, Brack et al. 2007) e de pesticidas ditos "benignos" (*e.g.*, Gárcia de Llasera & Bernal-González 2001, Chelme-Ayala et al. 2005, Guest et al. 2006, Wilson & Foos 2006). Relativamente à realidade portuguesa, os trabalhos desta natureza não são tão abundantes. No entanto, é também frequente a detecção de pesticidas em várias matrizes do compartimento aquático. Numa amostragem extensa de águas superficiais em todo o território, Tauler et al. (2001) detectaram vários pesticidas em quantidades largamente acima dos limites impostos pela UE relativamente à água para consumo humano. Em águas subterrâneas da Beira Litoral e do Ribatejo, Batista et al. (2002) detectaram quantidades preocupantes de variados resíduos de pesticidas em amostras de poços para irrigação. Cerejeira et

al. (2003) registaram uma grande diversidade de resíduos de pesticidas organoclorados e organofosforados, entre outros de maior especificidade, nalguns casos em concentrações consideráveis, em várias massas de água superficiais e subterrâneas portuguesas. Em 62% das 171 amostras de água recolhidas de poços em orizicultura da região do Baixo Sado, foram detectados diversos resíduos de pesticidas, entre os quais 3,4 – dicloroanilina, clorfenvinfos, molinato, propanil, tendo em alguns casos sido detectados níveis acima de 0,1µg/L (Silva et al. 2006). Em reservatórios no Alentejo foi registada a presença de compostos fenólicos associados à utilização de pesticidas (Barrico et al. 2006) e, muito recentemente, foram detectados diversos pesticidas e seus metabolitos em águas subterrâneas de uma zona a Noroeste do país com prática hortícola intensiva, frequentemente em concentrações acima dos limites estabelecidos pela UE (Gonçalves et al. 2007).

Os programas de monitorização química de pesticidas em matrizes aquáticas constituem um passo relevante na avaliação do nível de contaminação dos ecossistemas e uma acção fundamental para a protecção desses mesmos ecossistemas. No entanto, se estes programas fornecem informações fundamentais ao nível da exposição provável a que os organismos possam estar sujeitos, não permitem conclusões fiáveis acerca dos efeitos adversos que essa exposição poderá potenciar nas comunidades bióticas. Isto é, embora a monitorização puramente química dos ecossistemas permita um rastreio dos contaminantes existentes, não facilita a identificação desses contaminantes como poluentes efectivos (*All pollutants are contaminants, but not all contaminants are pollutants*; Campbell et al. 2002, Chapman 2007). É precisamente neste contexto que se desenvolvem os procedimentos de análise de risco ecológico, pressupondo que o risco de um determinado contaminante resultará da interacção entre a exposição e os efeitos (SETAC 1997). É também nesta interacção que assentam as recomendações para procedimentos de análise de risco *standard* e as normas para a autorização da colocação de pesticidas no mercado comum da UE (CE 1991, EC 2002b). Os efeitos de contaminantes, que deverão ser considerados inaceitáveis no contexto da sustentabilidade e protecção dos ecossistemas aquáticos, reconhecem-se, por exemplo, na redução da biodiversidade e nos impactos adversos ao nível da função do ecossistema (EC 2002b). Perante estas directrizes, o grande desafio da investigação em ecotoxicologia acaba por assentar no desenvolvimento de metodologias de análise que possam discriminar os efeitos dos contaminantes em ecossistemas aquáticos, tendo em conta os diversos níveis biológicos a que estes, dadas as suas propriedades físico-químicas, podem actuar (FIGURA I.4). Os pesticidas são químicos com potencial para serem estudados neste contexto, o que está relacionado com as

propriedades essenciais que asseguram o seu sucesso como produto fitofarmacêutico. Além de serem xenobióticos capazes de afectar todos os grupos taxonómicos do biota, incluindo organismos não-alvo, a vários níveis de organização, geralmente são desenvolvidos para serem minimamente resistentes à degradação ambiental, o que favorece o seu potencial para exercer efeitos tóxicos a curto e a longo-prazo.

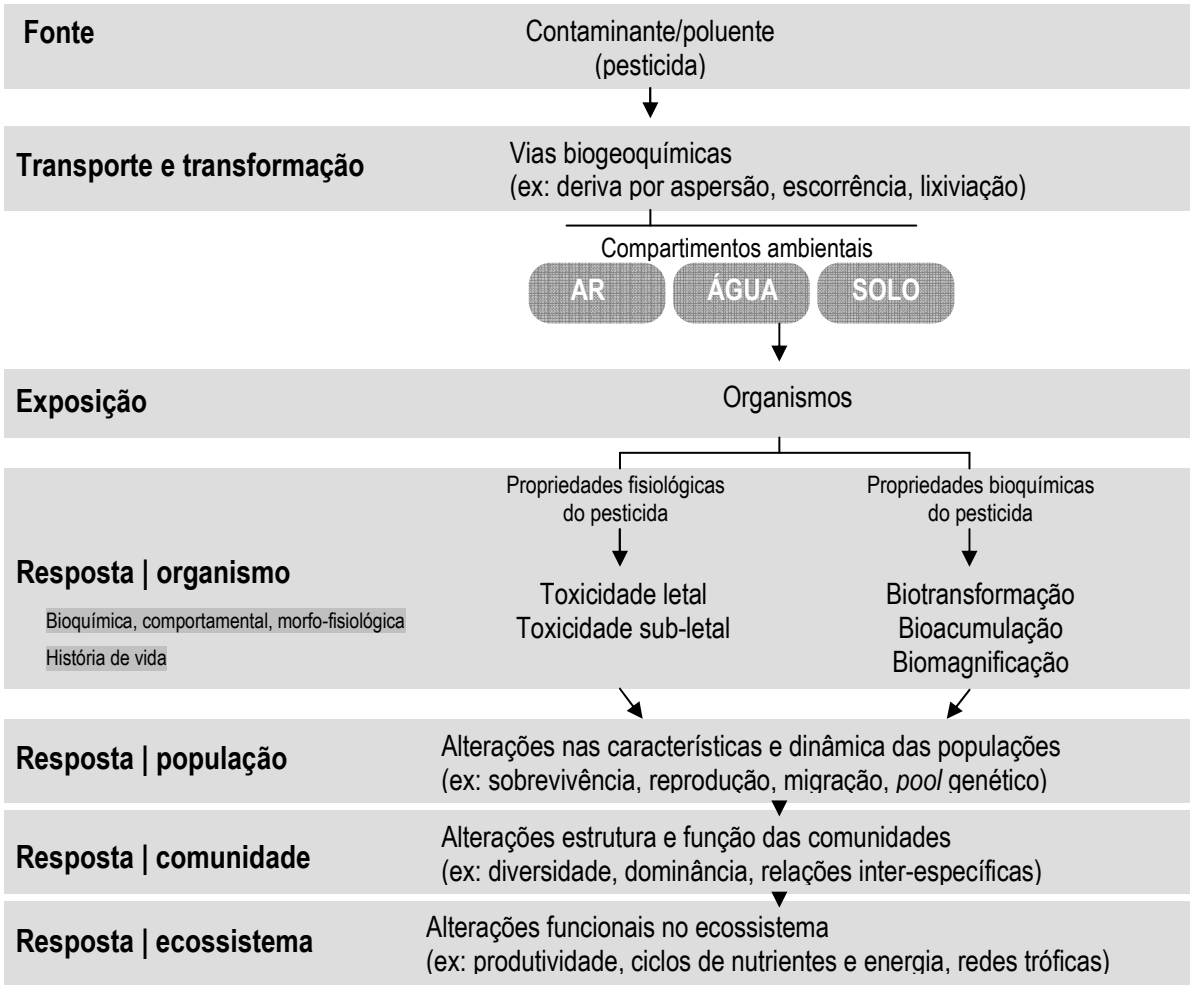


FIGURA I.4 | Diagrama representativo das vias principais com as quais se identifica o impacto potencial dos pesticidas no ecossistema aquático, entendido como um todo ou considerando os seus componentes bióticos principais (adaptado de Connel & Miller 1984 e Gerhardt 2007).

A Ecotoxicologia, enquanto ramo híbrido entre a Ecologia e a Toxicologia, reunirá talvez as melhores condições para se assumir como contexto da avaliação dos efeitos dos pesticidas nos ecossistemas, aos vários níveis a que essa avaliação se pode processar (FIGURA I.4). Num passado

não muito longínquo, os ecólogos dedicavam-se exclusivamente à mecânica da influência de factores bióticos e abióticos na distribuição das espécies ou nas suas interacções, sendo que os toxicólogos se focavam apenas em avaliações estanques de toxicidade; o desenvolvimento da Ecotoxicologia (geralmente atribuído aos anos 60) permitiu a fluência de conhecimentos e técnicas entre as duas áreas, tendo gerado as condições necessárias para uma visão integradora da avaliação de potenciais efeitos de contaminantes, a diversos níveis do ecossistema (Relyea & Hoverman 2006). Neste contexto, e de acordo com o nível de análise e com as hipóteses de trabalho, a literatura regista uma larga gama de estudos que seguem abordagens variadas, desde as puramente laboratoriais às semi-naturais e até às que abordam holisticamente as condições ambientais naturais (revisões por Hanazato 2001, Stark & Banks 2003, van Wijngaarden et al. 2005 e Relyea & Hoverman 2006).

De facto, ao longo das várias etapas ecotoxicológicas de análise do risco ecológico de um determinado pesticida, podem ser utilizadas metodologias que se enquadram nos diversos níveis de organização de um ecossistema, assim obtendo um dossier de informação integrada e pormenorizada que permite estimar, com elevado grau de certeza, esse mesmo risco (Maltby 2006). Por exemplo, (i) Robbens et al. (2007) advoga as potencialidades das técnicas de monitorização da transcrição genética na discriminação do perfil toxicológico, do modo de acção e dos efeitos de xenobióticos em organismos não-alvo; (ii) Chambers et al. (2002) contextualiza a relevância que podem assumir os biomarcadores de exposição e de efeitos, que podem fornecer informações relevantes sobre a história de exposição a contaminantes a que um organismo esteve sujeito ou sobre o modo de acção específico do químico nesse organismo, respectivamente; (iii) Calow et al. (1997) discutem a relevância dos testes de toxicidade aguda e crónica, que abordam os efeitos letais e sub-letais dos contaminantes ao nível do indivíduo, constituindo por isso plataformas de base, úteis e relativamente simplificadas, para análises mais abrangentes a níveis funcionais de maior complexidade no ecossistema (e.g., população, comunidade); (iv) Van den Brink et al. (2005) reconhecem os microcosmos como sistemas de teste, que se posicionam entre os testes ao nível individual e os testes de mesocosmos, de campo ou as avaliações holísticas de comunidades, capazes de algum poder discriminatório acerca de alterações estruturais e funcionais que possam ser induzidas por um determinado xenobiótico num ecossistema; (v) De Jong et al. (2005) revêem as recomendações da Comunidade Europeia e propõem um conjunto de metodologias e técnicas, características de fases avançadas de uma análise de risco ecológico, a aplicar em contexto experimental de mesocosmos ou de campo. Naturalmente que, quanto mais abrangente é a

metodologia de avaliação ecotoxicológica utilizada, maior será o grau de relevância ecológica da análise mas menor será, por outro lado, a especificidade e resolução dos resultados, bem como o grau de compreensão mecanística da resposta obtida.

É na interface entre a resposta ao nível do organismo e ao nível da população (de acordo com a FIGURA I.4) que se centra a avaliação de efeitos de dois pesticidas em *Daphnia* efectuada no decorrer deste trabalho. A toxicidade do herbicida Propanil e do insecticida Metomil, pesticidas pertencentes a classes químicas distintas e com modos de acção muito específicos e claramente distintos, foi avaliada, utilizando parâmetros ecotoxicológicos sub-celulares, individuais e populacionais. Naturalmente que os zooplanctontes não são o alvo generalista de nenhum dos dois pesticidas; no entanto, mesmo tendo em conta a especificidade dos seus modos de acção, é provável que haja coincidência de alguns receptores sub-celulares e a consequente interferência em vias metabólicas diversas, características dos organismos não-alvo. Neste sentido, torna-se relevante apresentar uma breve caracterização dos pesticidas em causa.

Caracterização geral do herbicida Propanil

O herbicida Propanil pertence ao grupo químico das anilidas e é reconhecido pela IUPAC através da designação 3',4'-dicloropropionanilida (FIGURA I.6). Trata-se de um pesticida de superfície ou de contacto foliar, largamente utilizado desde 1961 no período de pós-emergência do arroz para controlar ervas infestantes (Tomlin 2001). Aparentemente, as propriedades físico-químicas favorecem a sua mobilidade através do solo, facilitando a contaminação do compartimento aquático pelos seus resíduos (*vide* Capítulo III). Foi já por diversas vezes detectado em massas de água superficiais e subterrâneas essencialmente associadas a produções de arroz (*e.g.*, Albanis et al. 1998, Perera et al. 1999, Batista et al. 2002, Cerejeira et al. 2003, Silva et al. 2006, Gonçalves et al. 2007). O Propanil é um químico muito específico e selectivo quanto ao modo de acção, actuando por inibição do transporte de electrões ao nível do receptor do fotossistema II (Tomlin 2001), nas espécies vegetais sensíveis (geralmente, é o caso das ervas infestantes). Ao contrário das espécies em produção, as espécies sensíveis possuem quantidades residuais da enzima aril-acilamidase, que catalisa a metabolização do Propanil

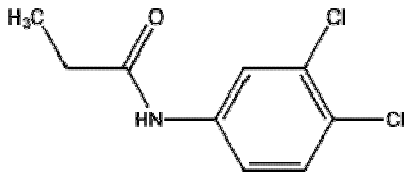


FIGURA I.6 | Estrutura molecular do Propanil (Fonte: Kegley & Orme 2007)

para os seus metabolitos primários (3,4-dicloroanilina e ácido propiónico) desactivando-o (Frear & Still 1968, Yih et al. 1968). Assim, as espécies sensíveis não conseguem contrariar a intervenção do propanil no bloqueio da fotossíntese. O propanil é um composto estável, sendo que a sua degradação primária resulta de hidrólise exclusivamente enzimática, ou seja, trata-se de um processo que é sempre mediado biologicamente (Mitsou et al. 2006). A destoxificação nas espécies vegetais em produção (*e.g.*, arroz, trigo) prossegue com a metabolização do ácido propiónico para dióxido de carbono via β-oxidação (Still 1968a) e conjugação da dicloranilina com glicose ou outros sacarídeos (Still 1968b). Os insecticidas organofosforados e carbamatos são inibidores competitivos da actividade enzimática da aril-acilamidase, o que é por vezes aproveitado, sob condições controladas, para aumentar a eficácia do controlo das ervas infestantes (Frear & Still 1968, Carey III et al. 1997). Apesar de se tratar de um pesticida desenhado para interferir com vias metabólicas específicas dos vegetais, foi já demonstrada a sua capacidade para induzir efeitos deletérios em animais e especificamente em cladóceros (*e.g.*, Moore & Farris 1997, Moore et al. 1998, Villarroel et al. 2003, Kegley & Orme 2007)

Caracterização geral do insecticida Metomil

O insecticida Metomil pertence ao grupo químico dos carbamatos. Trata-se de um carbamato mono-metílico, um composto sintético reconhecido pela IUPAC através da designação química " S-metil N-(metilcarbamoiloxil) tioacetimidado" (FIGURA I.7). O metomil é um princípio activo com penetração por contacto e com largo espectro de acção, utilizado sob várias formulações comerciais amplamente comercializadas desde 1967, e utilizadas para controlar uma gama larga de espécies, sobretudo de insectos em produções variadíssimas de fruta, vegetais, plantas ornamentais e cereais (Tomlin 2001). As propriedades físico-químicas e as técnicas de aplicação que são utilizadas favorecem o potencial deste pesticida para contaminar o compartimento aquático (*vide* Capítulo IV) e, de facto, já foi registada a presença deste pesticida em matrizes aquáticas (*e.g.*, Barceló et al. 1996, García de Llasera & Bernal-González 2001, Wilson & Foos 2006). Os carbamatos actuam directamente no local de acção sendo que geralmente não é necessário qualquer processo de bioactivação. O seu modo de acção passa pela inibição reversível da

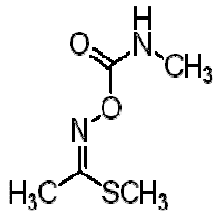


FIGURA I.7 | Estrutura molecular do Metomil (Fonte: Kegley & Orme 2007)

acetilcolinesterase (AChE) através da N-metilcarbamilação do grupo hidroxil-serina da enzima, com a consequente destruição do carbamato e formação de um fenol ou uma oxima (Hutson & Roberts 1985). Sendo a inibição da ACHE pelo metomil uma reacção reversível, é expectável que haja uma recuperação total dos organismos após a exposição ao tóxico. Andersen et al. (2006), embora trabalhando com um outro carbamato, demonstraram que a resposta não é tão linear: de facto, após uma primeira exposição aguda, seguida de transferência para meio pristino, *Daphnia* recupera a mobilidade, mas surpreendentemente a sua condição geral não regressará aos níveis normais e um segundo pulso de pesticida, mesmo que mais fraco, é fatal. A inibição da ACHE resulta na inibição da hidrólise e consequente acumulação do neurotransmissor acetilcolina (ACh) nos receptores pós-sinápticos e nas junções neuromusculares do sistema nervoso central e periférico, promovendo em geral uma hiper-activação dos tecidos musculares e consequentes efeitos ao nível funcional e comportamental dos organismos e, em última instância, morte por asfixia (Roex et al. 2003, Rickwood & Galloway 2004). Os efeitos nos organismos aquáticos, sejam eles vertebrados ou invertebrados, são obrigatoriamente diferentes, até porque, logo que haja passagem de água através do sistema branquial, o oxigénio é transferido e a asfixia será um efeito pouco verosímil. Por outro lado, o sistema de neurotransmissão nos invertebrados não está tão claramente definido como o dos vertebrados, e efectivamente não há evidências de uma relação linear entre a inibição de ACHE (biomarcador sub-celular) e as respostas biológicas individuais (ao nível do organismo) em exposições de *Daphnia* a carbamatos (Printes & Callaghan 2004). A inibição de outras esterases pelos carbamatos, bem como variações nos mecanismos enzimáticos podem explicar esta observação (Barata et al. 2004, Printes & Callaghan 2004), alargando as perspectivas relativas aos mecanismos associados à toxicidade destes pesticidas em *Daphnia*.

Objectivos e estrutura da dissertação

Considerando *Daphnia* como modelo fundamental em estudos ecotoxicológicos e como organismo de teste por excelência em processos de análise de risco ecológico relacionadas com o compartimento aquático, esta dissertação pretende constituir uma contribuição para a caracterização geral do conjunto de factores (naturais ou gerados pelo Homem) que podem condicionar as suas populações, interferindo ao nível do organismo e, como consequência, promovendo eventuais alterações a níveis mais elevados da organização biológica. Neste contexto – e procurando uma abordagem que pudesse gerar dados relevantes, por um lado passíveis de ser integrados em estudos mais generalizados/padronizados e, por outro, capazes de delinear cenários mais relevantes na realidade europeia/mediterrânica –, foi utilizada, na generalidade dos trabalhos, uma espécie padronizada (*Daphnia magna*) e três populações indígenas (*Daphnia* cf *longispina*) isoladas a partir de amostras recolhidas em lagos/reservatórios portugueses.

Assim, numa primeira fase, seleccionou-se a disponibilidade alimentar como factor natural de *stress* já que condiciona, logo à partida, a capacidade energética do organismo para desempenhar todas as funções vitais. Com esta abordagem pretendeu-se analisar especificamente as respostas individuais e populacionais das várias populações de *Daphnia*, quando sujeitas a variações na quantidade de alimento disponível, e avaliar até que ponto o tamanho corporal pode ser entendido como o traço somático fundamental na determinação dessas mesmas respostas (Capítulo II).

A abordagem que se seguiu visou especificamente a avaliação detalhada de efeitos, ao nível individual e populacional nas diferentes populações de *Daphnia*, de dois químicos sintéticos, e pretendeu analisar a influência que a disponibilidade de alimento pode ter no condicionamento das respostas ao tóxico. Foram escolhidos para o estudo o herbicida Propanil (Capítulo III) e o insecticida Metomil (Capítulo IV).

Por fim, e considerando a falta de literatura relacionada com as vias metabólicas essenciais envolvidas na acção destes dois pesticidas em organismos não-alvo como *Daphnia*, foi desenvolvido um trabalho relacionado com a expressão genética associada à exposição destes organismos a cada um dos químicos (Capítulo V). O objectivo fundamental deste estudo foi

precisamente a discriminação dos efeitos dos pesticidas a nível sub-celular, já que a técnica molecular utilizada permite esse nível de detalhe em associação com exposições controladas.

Tal como é sugerido pela discriminação anterior dos objectivos propostos, esta dissertação organiza-se por capítulos. A seguir a um primeiro capítulo onde é feita uma introdução geral e a revisão da literatura relevante para o enquadramento do trabalho, apresentam-se quatro capítulos, correspondentes aos objectivos específicos delineados e reflectindo o trabalho experimental efectuado, que possuem um carácter de relativa independência entre eles. Assim, cada um destes capítulos é composto pelas seguintes secções próprias: introdução, material e métodos, resultados, discussão, por vezes conclusões, e referências. Esta opção de apresentação reflecte a preparação de cada capítulo para publicação em revistas científicas internacionais, razão pela qual o texto é mantido na sua versão em língua inglesa. O último capítulo da dissertação integra e sintetiza a informação gerada ao longo do trabalho, abordando o seu enquadramento à luz do actual nível de conhecimento científico nas áreas específicas exploradas.

REFERÊNCIAS BIBLIOGRÁFICAS

Abrantes N, Antunes SC, Pereira MJ & Gonçalves F (2006). Seasonal succession of cladocerans and phytoplankton and their interaction in a shallow eutrophic lake (Lake Vela, Portugal). *Acta Oecologica* 29: 54-64.

Albanis TA, Hela DG, Sakellarides TM & Konstantinou IK (1998). Monitoring of pesticides residues and their metabolites in surface and underground waters of Imathia (N. Greece) by means of solid-phase extraction disks and gas chromatography. *Journal of Chromatography A* 823: 59-71.

Alonso M (1996). Crustacea – Branchiopoda. *In*: MAR Sánchez (coord. ed.), Fauna Iberica, Vol. 7. Museo Nacional de Ciencias Naturales, CSIC, Madrid. 486p.

Andersen TH, Tjørnhøj R, Wollenberger L, Slothuus T & Baun A (2006). Acute and chronic exposure of *Daphnia magna* to Dimethoate and Pirimicarb. *Environmental Toxicology and Chemistry* 25: 1187-1195.

Baird DJ, Barber I, Bradley, M, Calow P & Soares AMVM (1989). The Daphnia bioassay: a critique. *Hydrobiologia* 188/189: 403-406.

Barata C, Solayan A & Porte C (2004). Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. *Aquatic Toxicology* 66: 125-139.

Barceló D, Chiron S, Fernandez-Alba A, Valverde A & Alpendurada MF (1996). Monitoring pesticides and metabolites in surface water and groundwater in Spain. *Herbicide metabolites in surface water and groundwater ACS Symposium Series* 630: 237-253.

Barr DB & Needham LL (2002). Analytical methods for biological monitoring of exposure to pesticides: a review. *Journal of Chromatography B* 778: 5-29.

Barrico ML, Nabais C, Martins MJ & Freitas H (2006). Sources of phenolic compounds in two catchments of southern Portugal – effect of season, land use and soil type. *Chemosphere* 65: 482-488.

Batista S, Silva E, Galhardo S, Viana P & Cerejeira MJ (2002). Evaluation of pesticide contamination of ground water in two agricultural areas of Portugal. *International Journal of Environmental Analytical Chemistry* 82: 601-609.

Baudo R (1987). Ecotoxicological testing with *Daphnia*. *Memorie dell' Istituto Italiano di Idrobiologia* 45: 461-482.

Becker C & Boersma M (2003). Resource quality effects on life histories of *Daphnia*. *Limnology & Oceanography* 48: 700-706.

Becker C & Boersma M (2005). Differential effects of phosphorous and fatty acids on *Daphnia magna* growth and reproduction. *Limnology & Oceanography* 50: 388-397.

Benzie JAH (2005). Cladocera: the Genus *Daphnia* (including Daphniopsis). *In*: HJF Dumont (coord. ed.), Guides to the Identification of the microinvertebrates of the continental waters of the world, Vol. 21. Kenoby Productions, Ghent & Backhuys Publishers, Leiden. 376p.

Boersma M (1995). The allocation of resources to reproduction in *Daphnia galeata*: against the odds? *Ecology* 76: 1251-1261.

Boersma M (1997). Offspring size in *Daphnia*: does it pay to be overweight? *Hydrobiologia* 360: 79-88.

Boersma M, Boriss H & Mitchell SE (2000). Maternal effects after sexual reproduction in *Daphnia magna*. *Journal of Plankton Research* 22: 279-285.

Boersma M & Vijverberg J (1994). Seasonal variation in the condition of two *Daphnia* species and their hybrid in a eutrophic lake: evidence for food limitation. *Journal of Plankton Research* 16: 1793-1809.

Braband A, Richter S, Hiesel R & Scholtz G (2002). Phylogenetic relationships within the Phyllopoda (Crustacea, Branchiopoda) based on mitochondrial and nuclear markers. *Molecular Phylogenetics and Evolution* 25: 229-244.

Brack W, Klamer HJC, de Alda ML, Barceló D (2007). Effect-directed analysis of key toxicants in European river basins: a review. *Environmental Science and Pollution Research* 14: 30-38.

Bradley MC, Perrin N & Calow P (1991). Energy allocation in the cladoceran *Daphnia magna* Straus, under starvation and refeeding. *Oecologia* 86: 414-418.

Brendonck L & De Meester L (2003). Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia* 491: 65-84.

Brock TCM, Lahr J & van den Brink PJ (2000a). Ecological risks of pesticides in freshwater ecosystems – part 1: Herbicides. Alterra-Rapportt 088. Alterra, Green World Research, Wageningen. 124p.

Brock TCM, van Wijngaarden RPA & van Geest GJ (2000b). Ecological risks of pesticides in freshwater ecosystems – part 2: Insecticides. Alterra-Rapportt 089. Alterra, Green World Research, Wageningen. 142p.

Brooks JL & Dodson SI (1965). Predation, body size and composition of plankton. *Science* 150: 28-35.

Brown CD, Carter AD & Holis JM (1995). Soils and pesticide mobility. *In:* TR Roberts & PC Kearney (Eds.), *Environmental behaviour of agrochemicals* (Vol. 9 – Series: Progress in pesticide biochemistry and technology). John Wiley & Sons, West Sussex. pp 131-179.

Calow P, Sibly RM & Forbes V (1997). Risk assessment on the basis of simplified life-history scenarios. *Environmental Toxicology and Chemistry* 16: 1983-1989.

Campbell PGC, Chapman PM & Hale BA (2002). Risk assessment of metals in the environment. *In*: RE Hester & RM Harrinson (Eds.), *Chemicals in the environment: assessing and managing risk*. (nº 22 – Series: Issues in Environmental Science and Technology). Royal Society of Chemistry, Cambridge, pp 102-131.

Caramujo MJ & Boavida MJ (2000). The crustacean communities of River Tagus reservoirs. Zooplankton structure as reservoir trophic state indicator. *Limnetica* 18: 37-56.

Carey III VF, Hoagland RE & Talbert RE (1997). Resistance mechanism of Propanil-resistant barnyardgrass: II. In-vivo metabolism of the Propanil molecule. *Pesticide Science* 49: 333-338.

Carpenter SR, Kitchell JF & Hodgson JR (1985). Cascading trophic interactions and lake productivity: fish predation and herbivory can regulate lake ecosystems. *Bioscience* 35: 634-639.

Caruso BS 2001. Risk-based targeting of diffuse contaminant sources at variable special scales in a New Zealand high country catchment. *Journal of Environmental Management* 63: 249-268.

Carter A (2000). How pesticides get into water – and proposed reduction measures. *Pesticide Outlook*: 149-156.

Castro BB (2007). Ecologia e selecção de habitat em crustáceos zooplanctónicos de lagos pouco profundos. Tese de Doutoramento. Universidade de Aveiro, Aveiro. 168p.

Castro BB, Consciência S & Gonçalves F (2007). Life-history responses of *Daphnia longispina* to mosquitofish (*Gambusia holbrooki*) and pumpkinseed (*Lepomis gibbosus*) kairomones. *Hydrobiologia* 594: 165-174.

Castro M, Silva-Ferreira AC, Manaia CM, Nunes OC (2005). A case study of molinate application in a Portuguese rice field: herbicide application and proposal of a clean-up methodology. *Chemosphere* 59: 1059-1065.

CE (1991). Directiva 91/414/EEC do Conselho de 15 de Junho de 1991 relativa à colocação dos produtos fitofarmacêuticos no mercado. Jornal Oficial das Comunidades Europeias L230 de 19.08.1991: 1-32.

CE (1998). Directiva 98/83/CE do Conselho de 3 de Novembro de 1998 relativa à qualidade da água destinada ao consumo humano. Jornal Oficial das Comunidades Europeias L330 de 05.12.1998: 32-54.

CE (2005). Regulamento (CE) nº 396/2005 do Parlamento Europeu e do Conselho de 23 de Fevereiro de 2005. Jornal Oficial das Comunidades Europeias L70 de 16.03.2005: 1-16.

CE (2006). Regulamento (CE) nº 1907/2006 do Parlamento Europeu e do Conselho de 18 de Dezembro de 2006. Jornal Oficial da União Europeia L396 de 30.12.2006: 1-854.

Cerejeira MJ, Viana P, Batista S, Pereira T, Silva E, Valério MJ, Silva A, Ferreira M & Silva-Fernandes AM (2003). Pesticides in Portuguese surface and ground waters. *Water Research* 37: 1055-1063.

Chambers JE, Boone JS, Carr RL, Chambers HW & Straus DL (2002). Biomarkers as predictors in health and ecological risk assessment. *Human and Ecological Risk Assessment* 8: 165-176.

Chapman PE (2007). Determining when contamination is pollution – weight of evidence determinations for sediments and effluents. *Environment International* 33: 492-501.

Chelme-Ayala P, El-Din MG, Smith DW & Guest RK (2005). Pesticides and herbicides. *Water Environment Research* 77: 2021-2129.

Cohen SZ, Wauchope RD, Klein AW, Eadsforth CV & Graney R (1995). Offside transport of pesticides in water: mathematical models of pesticide leaching and runoff (technical report). *Pure and Applied Chemistry* 67: 2109-2148.

Connel DW & Miller GJ (1984). Chemistry and Ecotoxicology of pollution. John Wiley, New York. 444p.

Craven A (2002). New development in environmental exposure assessment in the EU pesticide regulatory process. *International Journal of Environmental Analytical Chemistry* 82: 645-649.

D'Arcy B & Frost A (2001). The role of best management practices in alleviating water quality problems associated with diffuse pollution. *The Science of the Total Environment* 265: 359-367.

Declerck S & De Meester L (2003). Impact of fish predation on coexisting *Daphnia* taxa: a partial test of the temporal hybrid superiority hypothesis. *Hydrobiologia* 500: 83-94.

De Jong FMW, Mensink BJWGH, Smit CE & Montforts MHMM (2005). Evaluation of ecotoxicological field studies for authorization of plant protection products in Europe. *Human and Ecological Risk Assessment* 11: 1157-1176.

De Meester L (1996). Local genetic differentiation and adaptation in freshwater zooplankton populations: Patterns and processes. *Écoscience* 3: 385-399.

De Meester L, Cousyn C & Vanoverbeke J (1998). Chemical interactions, maternal effects and the hatching of *Daphnia* diapausing eggs. *Archives für Hydrobiologie* 52: 263-272.

De Meester L, Vanoverbeke J, De Gelas K, Ortellis R & Spaak P (2006). Genetic structure of cyclical parthenogenetic zooplankton populations – a conceptual framework. *Archives für Hydrobiologie* 167: 217-244.

DeMott WR (1982). Feeding selectivities and relative ingestion rates of *Daphnia* and *Bosmina*. *Limnology & Oceanography* 27: 518-527.

DeMott WR, Gulati RD & Van Donk E (2001). *Daphnia* food limitation in three hypereutrophic Dutch lakes: Evidence for exclusion of large-bodied species by interfering filaments of cyanobacteria. *Limnology & Oceanography* 46: 2054-2060.

Deng H-W (1996). Environmental and genetic control of sexual reproduction in *Daphnia*. *Heredity* 76: 449-458.

Deng H-W & Lynch M (1996). Change of genetic architecture in response to sex. *Genetics* 143: 203-212.

Ebert D (1991). The effect of size at birth, maturation threshold and genetic differences on the life-history of *Daphnia magna*. *Oecologia* 86: 243-250.

Ebert D (1993). The trade-off between offspring size and number in *Daphnia magna*: the influence of genetic, environmental and maternal effects. *Archives für Hydrobiologie-Supplement* 90: 453-473.

EC (2002a). Guidance Document on Terrestrial Ecotoxicology – under Council Directive 91/414/EEC. European Comission – Heath & Consumer Protection Directorate-General.

EC (2002b). Guidance Document on Aquatic Ecotoxicology – in the context of the Directive 91/414/EEC. European Comission – Heath & Consumer Protection Directorate-General.

EC (2007). The use of Plant Protection Products in the European Union – data 1992-2003. European Comunities – Eurostat, Luxembourg, 222p.

Edmondson WT (1987). *Daphnia* in experimental ecology: notes on historical perspectives. *Memorie dell' Istituto Italiano di Idrobiologia* 45: 11-30.

Epp GT (1996). Clonal variation in the survival and reproduction of *Daphnia pulicaria* under low-food stress. *Freshwater Biology* 35: 1-10.

Flury M, Leuenberger J, Studer B &Flühler (1995). Transport of anions and herbicides in a loamy and a sandy field soil. *Water Resources Research* 31: 823-835.

Flury M (1996). Experimental evidence of transport of pesticides through field soils – a review. *Journal of Environmental Quality* 25: 25-45.

Frear DS & Still GG (1968). The metabolism of 3',4'-propionanilide in plants. Partial purification and properties of an aryl acylamidase from rice. *Phytochemistry* 7: 913-920.

Fryer G (1987). A new classification of the branchiopod Crustacea. *Zoological Journal of the Linnean Society* 91: 357-383.

Gabriel W (1982). Modelling reproductive strategies of *Daphnia*. *Archives für Hydrobiologie* 95: 69-80.

García de Llasera MP & Bernal-González M (2001). Presence of carbamate pesticides in environmental waters from the Northwest of Mexico: determination by liquid chromatography. *Water Research* 35: 1933-1940.

Geraldes AM & Boavida MJ (2004). What factors affect the pelagic cladocerans of the meso-eutrophic Azibo reservoir? *Annales de Limnologie* 40: 101-111.

Gerhardt A (2007). Aquatic behavioral Ecotoxicology – prospects and limitations. *Human and Ecological Risk Assessment* 13: 481-491.

Ghadouani A, Pinel-Alloul B & Prepas EE (2007). Could increased cyanobacterial biomass following forest harvesting cause a reduction in zooplankton body size structure? *Canadian Journal of Fisheries and Aquatic Sciences* 63: 2308-2317.

Gießler S, Mader E & Schwenk K (1999). Morphological evolution and genetic differentiation in *Daphnia* species complexes. *Journal of Evolutionary Biology* 12: 710-723.

Glazier DS (1992). Effects of food, genotype and maternal size and age on offspring investment in *Daphnia magna*. *Ecology* 73: 910-926.

Glazier DS (1998). Does body storage act as food availability cue for adaptative adjustment of egg size and number in *Daphnia magna*? *Freshwater Biology* 40: 87-92.

Gliwicz ZM (1990). Food thresholds and body size in cladocerans. *Nature* 343: 638-640.

Gliwicz ZM (2001). Species-specific population-density thresholds in cladocerans? *Hydrobiologia* 442: 291-300.

Golfinopoulos SK, Nikolaou AD, Kostopoulou MN, Xilourgidis NK, Vagi MC & Lekkas DT (2003). Organochlorine pesticides in the surface waters of Northern Greece. *Chemosphere* 50: 507-516.

Gliwicz ZM & Guisande C (1992). Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia* 91: 463-467.

Gonçalves CM, Esteves da Silva JCG & Alpendurada MF (2007). Evaluation of the pesticide contamination of groundwater sampled over two years from a vulnerable zone in Portugal. *Journal of Agricultural and Food Chemistry* 55: 6227-6235.

Guest RK, Ikehata K, El-Din MG, Smith DW (2006). Pesticides and Herbicides. *Water Environment Research* 78: 1755-1801.

Guisande C & Gliwicz ZM (1992). Egg size and clutch size in two *Daphnia* species grown at different food levels. *Journal of Plankton Research* 14: 997-1007.

Gurney WSC, McCauleu E, Nisbet RM & Murdoch WW (1990). The physiological ecology of *Daphnia*: a dynamic model of growth and reproduction. *Ecology* 71: 716-732.

Gyllström M & Hansson L-H (2004). Dormancy in freshwater zooplankton: Induction, termination and the importance of benthic-pelagic coupling. *Aquatic Sciences* 66: 274-295.

Hall DJ, Threlkeld ST, Burns CW & Crowley PH (1976). The size-efficiency hypothesis and the size structure of zooplankton communities. *Annual Review of Ecology and Systematics* 7: 177-203.

Hanazato T (2001). Pesticide effects on freshwater zooplankton: na ecological perspective. *Environmental Pollution* 112: 1-10.

Havel JE & Shurin JB (2004). Mechanisms, effects and scales of dispersal in freshwater zooplankton. *Limnology & Oceanography* 49: 1229-1238.

Hebert PDN (1987). Genetics of *Daphnia*. *Memorie dell' Istituto Italiano di Idrobiologia* 45: 439-460.

Hobæk A & Larsson P (1990). Sex determination in *Daphnia magna*. *Ecology* 71: 2255-2268.

Huber A, Bach M & Frede HG (2000). Pollution of surface waters with pesticides in Germany: modelling non-point source inputs. *Agriculture, Ecosystems and Environment* 80: 191-204.

Hülsmann S, Vijverberg J, Boersma M & Mooij WM (2004). Effects of infochemicals released by gape-limited fish on life-history traits of *Daphnia*: a maladaptive response? *Journal of Plankton Research* 26: 535-543.

Hutson DH & Roberts TR (1985). Insecticides. *In*: DH Hutson & TR Roberts (Eds.), *Insecticides* (Vol 5 – Series: Progress in pesticide biochemistry and toxicology). John Wiley & Sons, West Sussex, pp 1-34.

Innes DJ (1997). Sexual reproduction of *Daphnia pulex* in a temporary habitat. *Oecologia* 111: 53-60.

Innes DJ & Singleton DR (2000). Variation in allocation to sexual and asexual reproduction among clones of cyclically parthenogenetic *Daphnia pulex* (Crustacea: Cladocera). *Biological Journal of the Linnean Society* 71: 771-787.

Kashian D & Dodson SI (2004). Effects of vertebrate hormones on development and sex determination in *Daphnia magna*. *Environmental Toxicology and Chemistry* 23: 1282-1288.

Kegley S, Hill B & Orme S (2007). PAN pesticides database. Pesticide action Network, North America, San Francisco (http://www.pesticideinfo.org).

Keller B & Spaak P (2004). Nonrandom sexual reproduction and diapausing egg production in a *Daphnia* hybrid species complex. *Limnology & Oceanography* 49: 1393-1400.

Keller B, Wolinska J, Tellenbach C & Spaak P (2007). Reproductive isolation keeps hybridizing *Daphnia* species distinct. *Limnology & Oceanography* 52: 984-991.

Kleiven OT, Larsson P & Hobæk A (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* 65: 197-206.

Koivisto S (1995). Is *Daphnia magna* an ecologically representative zooplankton species in toxicity tests? *Environmental Pollution* 90: 263-267.

Kooijman SALM (1986). Population dynamics on the basis of budgets. *In*: Metz, J.A.J. & Diekman, O. (eds.): The dynamics of physiologically structured populations. Springer Verlag, Berlin, pp. 266-297.

Korovchinsky NM (1997). On the history of studies on cladoceran taxonomy and morphology, with emphasis on early work and causes of insufficient knowledge of the diversity of the group. *Hydrobiologia* 360: 1-11.

Korovchinsky M (2006). The cladocera (Crustacea: Branchiopoda) as a relict group. *Zoological Journal of the Linnean Society* 147:109-124.

Korpelainen H (1986). The effects of temperature and photoperiod on life history parameters of *Daphnia magna* (Crustacea: Cladocera). *Freshwater Biology* 15: 615-620.

Korpelainen H (1990). Sex ratios and conditions required for environmental sex determination in animals. *Biological Reviews* 65: 147-184.

Kotov AA & Boikova OS (2001). Study of the late embryogenesis of *Daphnia* (Anomopoda, 'Cladocera', Branchiopoda) and a comparison of development in Anomopoda and Ctenopoda. *Hydrobiologia* 442: 127-143.

Kotov AA & Korovchinsky NM (2006). First record of fossil Mesozoic Ctenopoda (Crustacea: Cladocera). *Zoological Journal of the Linnean Society* 146: 269-274.

Kotov AA (2007). Jurassic Cladocera (Crustacea, Branchiopoda) with a description of an extinct Mesozoic order. *Journal of Natural History* 41: 13-37.

Kreutzer C & Lampert W (1999). Exploitative competition in differently sized *Daphnia* species: a mechanistic explanation. *Ecology* 80: 2348-2357.

Lair N (1991). Grazing and assimilation rates of natural populations of planktonic cladocerans in a eutrophic lake. *Hydrobiologia* 215: 51-61.

Lampert W (1978). A field study on the dependence of the fecundity of *Daphnia* spec. on food concentration. *Oecologia* 36: 363-369.

Lampert W (1987). Feeding and nutrition in *Daphnia*. *Memorie dell' Istituto Italiano di Idrobiologia* 45: 143-192.

Lampert W (2006). *Daphnia*: model herbivore, predator and prey. *Polish Journal of Ecology* 54: 607-620.

Lass S & Spaak P (2003). Chemically induced anti-predator defences in plankton: a review. *Hydrobiologia* 491: 221-239.

Leibold MA, Chase JM, Shurin JB & Downing AL (1997). Species turnover and the regulation of trophic structure. *Annual Review of Ecology and Systematics* 28:467-494.

Loague K, Corwin DL & Ellsworth TR (1998). The challenge of predicting non-point source pollution. *Environmental Science and Technology* 32: 130A-133A.

Lynch M (1977). Fitness and optimal body size in zooplankton populations. *Ecology* 58: 763-774.

Lynch M (1985). Speciation in the Cladocera. *Internationale Vereinigung fur theorische und angewandte Limnologie, Verhandlungen* 22: 3116-3123.

Lynch M (1989). The life-history consequences of resource depression in *Daphnia pulex*. *Ecology* 70: 246-256.

Lynch M (1992). The life-history consequences of resource depression in *Ceriodaphnia quadrangula* and *Daphnia ambigua*. *Ecology* 73: 1620-1629.

Lynch M & Gabriel W (1983). Phenotypic evolution and parthenogenesis. *The American Naturalist* 122: 745-764.

Lynch M & Gabriel W (1987). Environmental Tolerance. *The American Naturalist* 129: 283-303.

Maltby L (2006). Environmental Risk Assessment. *Issues in Environmental Science and Technology* 22: 84-101.

McCauley E, Murdoch WW & Nisbet RM (1990a). Growth, reproduction and mortality of Daphnia pulex Leydig: life at low food. *Functional Ecology* 4: 505-514.

McCauley E, Murdoch WW, Nisbet RM & Gurney WSC (1990b). The physiological ecology of *Daphnia*: development of a model of growth and reproduction. *Ecology* 71: 703-715.

McQueen DJ, Post JR & Mills EL (1986). Trophic relationships in freshwater pelagic ecosystems. *Canadian Journal of Fisheries and aquatic Sciences* 43: 1571-1581.

Miller TE, Burns JH, Munguia P, Walters EL, Kneitel JM, Richards PM, Mouquet N & Bouckley HL (2005). A critical review of twenty years' use of the Resource-Ratio Theory. *The American Naturalist* 165: 700-706.

Mitsou K, Koulianou A, Lambropoulou D, Pappas P, Albanis T & Lekka M (2006). Growth rate effects of antioxidant enzymes and metabolic fate of the herbicide Propanil in the aquatic plant *Lemna minor*. *Chemosphere* 62: 275-284.

Molles MC 1999. Ecology: Concepts and Applications. McGraw-Hill, EUA. 509p.

Moore MT & Farris JL (1997). Acute and chronic toxicity of the herbicide Stam® M-4 in field and laboratory exposures. *Archives of Environmental Contamination and Toxicology* 33: 199-202.

Moore MT, Pierce JR, Milam CD, Farris JL & Winchester EL (1998). Responses of non-target aquatic organisms to aqueous Propanil exposure. *Bulletin of Environmental Contamination and Toxicology* 61: 169-174.

Negrea S, Botnariuc N, Dumont HJ (1999). Phylogeny, evolution and classification of the Branchiopoda (Crustacea). *Hydrobiologia* 412: 191-212.

OECD (1998). *Daphnia magna* reproduction test - Test guideline 211. Organization for the Economic Cooperation and Development. Paris.

OECD (2004). OECD guideline for testing of chemicals 202 - *Daphnia* sp., acute immobilisation test. Organization for the Economic Cooperation and Development. Paris.

Olmstead AW & Le Blanc GA (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. *Environmental Toxicology and Chemistry* 19: 2107-2113.

Olmstead AW & LeBlanc (2007). The environmental-endocrine basis of gynandromorphism in a crustacean. *International Journal of Biological Sciences* 3: 77-84.

Oquist QA, Strock JS & Mulla DJ (2007). Influence of alternative and conventional farming practices on subsurface drainage and water quality. *Journal of Environmental Quality* 36: 1194-1204.

Pace ML (1984). Zooplankton community structure, but not biomass, influences the phosphorus-chlorophyll a relationship. *Canadian Journal of Fisheries and Aquatic Sciences* 41: 1089-1096.

Pauwels K, Stocks R, Verbiest A & DeMeester L (2007). Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*. *Hydrobiologia* 594: 91-96.

Pereira JL, Marques CR & Gonçalves F (2004). Allometric relations for *Ceriodaphnia* spp. and *Daphnia* spp. *Annales de Limnologie* 40: 11-14.

Perera A, Burleigh JR & Davis CB (1999). Movement and retention of Propanil (N-(3,4-dichlorophenyl)propanamide in a paddy-riverine wetland system in Sri Lanka. *Agriculture, Ecosystems and Environment* 72: 255-263.

Plath K & Boersma M (2001). Mineral limitation of zooplankton: stoichiometric constraints and optimal foranging. *Ecology* 82: 1260-1269.

Polishchuk LV & Vijverberg J (2005). Contribution analysis of body mass dynamics in *Daphnia*. *Oecologia* 144: 268-277.

Printes LB & Callaghan A (2004). A comparative study on the relationship between acetylcholinesterase activity and acute toxicity in *Daphnia magna* exposed to anticholinesterase insecticides. *Environmental Toxicology and Chemistry* 23: 1241-1247.

Reichenberger S, Bach M, Skitschak A & Frede H-G (2007). Mitigation strategies to reduce pesticide inputs into ground- and surface water and their effectiveness; a review. *The Science of the Total Environment* 384: 1-35.

Rellstab C & Spaak P (2007). Starving with a full gut? Effects of suspended particles on the fitness of *Daphnia hyalina*. *Hydrobiologia* 594: 131-139.

Relyea R & Hoverman J (2006). Assessing the ecology in ecotoxicology: a review and synthesis in freshwater ecosystems. *Ecology Letters* 9: 1157-1171.

Renner R (2002). The Kow controversy. *Environmental Science & Technology A-Pages* 36: 410A-413A.

Repka S (1997). Effects of food type on the life-history of *Daphnia* clones from lakes differing in trophic state. I. *Daphnia galeata* feeding on *Scenedesmus* and *Oscillatoria*. *Freshwater Biology* 37: 675-683.

Repka S (1998). Effects of food type on the life-history of *Daphnia* clones from lakes differing in trophic state. II. *Daphnia cucullata* feeding on mixed diets. *Freshwater Biology* 38: 685-692.

Reznick D (1993). New model systems for studying the evolutionary biology of aging: Crustacea. *Genetica* 91: 79-88.

Ribeiro ML, Lourencetti C, Pereira SY & Marchi MRR (2007). Contaminação de águas subterrâneas por pesticidas: avaliação preliminar. *Quimica Nova* 30: 688-694.

Rickwood CJ & Galloway TS (2004). Acetylcholinesterase inhibition as a biomarker of adverse effect – a study of Mytilus edulis exposed to the priority pollutant chlorfenvinphos. Aquatic toxicology 67: 45-56.

Rinke K & Vijverberg J (2005). A model approach to evaluate the effect of temperature and food concentration on individual life-history and population dynamics of *Daphnia*. *Ecological Modelling* 186: 326-344.

Robbens J, Van der Ven K, Maras M, Blust R & De Coen W (2007). Ecotoxicological risk assessment using DNA chips and cellular reporters. *TRENDS in Biotechnology* 25: 460-466.

Roex EWM, Keijzers R & Van Gestel CAM (2003). Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, *Danio rerio*, after chronic exposure to parathion. *Aquatic Toxicology* 64: 451-460.

Ruppert EE & Barnes RD (1994). Invertebrate Zoology, 6th ed. Saunders College Publishing, Fort Worth, EUA. 1056p.

Russell MH (1995). Recommended approaches to access pesticide mobility in soil. *In*: TR Roberts & PC Kearney (Eds.), *Environmental behaviour of agrochemicals* (Vol. 9 – Series: Progress in pesticide biochemistry and technology). John Wiley & Sons, West Sussex. pp 57-121.

Sarma SSS & Nandini S (2006). Review of recent ecotoxicological studies on cladocerans. *Journal of Environmental Science and Health B* 41: 1417-1430.

Schwenk K (1993). Interspecific hybridisation in *Daphnia*: distinction and origin of hybrid matrilines. *Molecular Biology and Evolution* 10: 1289-1302.

Schwenk K and Spaak P (1995). Evolutionary and ecological consequences of interspecific hybridisation in cladocerans. *Experientia* 51: 465-481.

Schwenk K, Sand A, Boersma M, Brehm M, Mader E, Offerhaus D & Spaak P (1998). Genetic markers, genealogies and biogeographic patterns in the Cladocera. Aquatic Ecology 32: 37-51.

Schwenk K, Posada D & Hebert PDN (2000). Molecular systematics of European *Hyalodaphnia*: the role of contemporary hybridization in ancient species. *Proceedings of the Royal Society of London B* 267: 1833-1842.

SETAC (1997). Technical issue paper – Ecological Risk Assessment. Society of Environmental Toxicology and Chemistry, Pensacola. 4p.

Silva E, Batista S, Viana P, Antunes P, Serôdio L, Cardoso AT & Cerejeira MJ (2006). Pesticides and nitrates in groundwater from oriziculture areas of the 'Baixo Sado' region (Portugal). *International Journal of Environmental Analytical Chemistry* 86: 955-972.

Sommer U & Stibor H (2002). Copepoda – Cladocera – Tunicata: the role of three major mesozooplankton groups in pelagic food webs. *Ecological Research* 17: 161-174.

Sommer U, Gliwicz ZM, Lampert W & Duncan A (1986). The PEG-model of seasonal succession of planktonic events in fresh waters. *Archives fur Hydrobiologie* 106: 433-471.

Spaak P (1995). Sexual reproduction in *Daphnia*: interspecific differences in a hybrid species complex. *Oecologia* 104: 501-507.

Spaak P (1996). Temporal changes in the genetic structure of the *Daphnia* species complex in Tjeukmeer, with evidence for backcrossing. *Heredity* 76: 539-548.

Spaak P & Hoekstra JR (1997). Fish predation on a *Daphnia* hybrid species complex: a factor explaining species coexistence? *Limnology & Oceanography* 42: 753-762.

Stark JD & Banks JE (2003). Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology* 48: 505-519.

Stibor H & Lampert W (2000). Components of additive variance in life-history traits of *Daphnia hyalina*: seasonal differences in the response to predator signals. *Oikos* 88: 129-138.

Still GG (1968a). Metabolic fate of 3',4'-dichloropropioanilide in plants: the metabolism of the propionic acid moiety. *Plant Physiology* 43: 543-546.

Still GG (1968b). Metabolism of 3',4'-dichloropropioanilide in plants: the metabolic fate of the 3',4'-dichloroaniline moiety. *Science* 159: 992-993.

Tauler R, De Almeida Azevedo D, Lacorte S, Céspedes R, Viana P & Barceló D (2001). Organic pollutants in surface waters from Portugal using chemometric interpretation. *Environmental Technology* 22: 1043-1054.

Taylor BE & Gabriel W (1985). Reproductive strategies of two similar *Daphnia* species. *Internationale Vereinigung fur theorische und angewandte Limnologie, Verhandlungen* 22: 3047-3050.

Taylor BE & Gabriel W (1992). To grow or not to grow: optimal resource allocation for *Daphnia*. *The American Naturalist* 139: 248-266.

Taylor BE & Gabriel W (1993). Optimal adult growth in a seasonal environment. *Functional Ecology* 7: 513-521.

Taylor DJ, Hebert PDN & Colbourne JK (1996). Phylogenetics and evolution of the *Daphnia longispina* group (Crustacea) based on 12S rDNA sequence and allozyme variation. *Molecular Phylogenetics and Evolution* 5: 495-510.

Taylor DJ, Crease TJ, Brown WM (1999). Phylogenetic evidence for a single long-lived clade of crustaceans cyclic parthenogens and its implications for the evolution of sex. *Proceedings of the Royal Society of London B* 266: 791-797.

Tessier AJ & Consolatti NL (1991). Resource quantity and offspring quality in *Daphnia*. *Ecology* 72: 468-478.

Tessier AJ & Goulden CE (1987). Cladoceran juvenile growth. *Limnology & Oceanography* 32: 680-686.

Threlkeld ST (1987). Daphnia life-history strategies and resource allocation patterns. *Memorie dell' Istituto Italiano di Idrobiologia* 45: 353-366.

Tillmann U & Lampert W (1984). Competitive ability of differently sized *Daphnia* species: an experimental test. *Journal of Freshwater Ecology* 2: 311-323.

Tilman D (1980). Resources: a graphical-mechanistic approach to competition and predation. *The American Naturalist* 116: 362-393.

Tomlin CDS (2001). *The pesticide manual*. British Crop Protection Council, Surrey. 1260p.

Tooby TE (1995). Assessing risk should not be a hazardous business. *In:* GA Best & AD Ruthven (eds.). *Pesticides – Developments, impacts, and controls*. The Royal Society of Chemistry, Cambridge. pp 142-149.

Trubetskova I & Lampert W (1995). Egg size and egg mass of *Daphnia magna*: response to food availability. *Hydrobiologia* 307: 139-145.

Trubetskova IL & Haney JF (2006). Effects of differing concentrations of microcystin-producing *Microcystis aeroginosa* on growth, reproduction, survivorship and offspring of *Daphnia magna*. *Archives für Hydrobiologie* 167: 533-546.

Van den Brink PJ, Tarazona JV, Solomon KR, Knacker T, Van den Brink NW, Brock TCM & Hoogland JPH (2005). The use of aquatic and terrestrial microcosms for the ecological risk assessment of veterinary medicinal products. *Environmental Toxicology and Chemistry* 24: 820-829.

Van Wijngaarden RPA, Brock TCM & Van den Brink PJ (2005). Threshold levels for effects of insecticides in freshwater ecosystems: a review. *Ecotoxicology* 14: 355-380.

Vanni MJ & Lampert W (1992). Food quality effects on life-history traits and fitness in the generalist herbivore *Daphnia*. *Oecologia* 92: 48-57.

Villarroel MJ, Sancho E, Ferrando MD & Andreu E (2003). Acute, chronic and sublethal effects of the herbicide propanil on *Daphnia magna*. *Chemosphere* 53: 857-864.

Wetzel RG (1993). *Limnologia* (trad. MJ Boavida). Fundação Calouste Gulbenkian, Lisboa, 919p.

Wilson PC & Foos JF (2006). Survey of carbamate and organophosphorous pesticide export from a South Florida (USA) agricultural watershed: implications of sampling frequency on ecological risk estimation. *Environmental Toxicology and Chemistry* 25: 2847-2852.

Yampolsky LY (1992). Genetic variation in the sexual reproduction rate within a population of a cyclical parthenogen, *Daphnia magna*. *Evolution* 46: 833-837.

Yih RY, McRae DH & Wilson HF (1968). Mechanism of selective action of 3',4'-dichloropropionanilide. *Plant Physiology* 43: 1291-1296.

Zaffagnini F (1987). Reproduction in *Daphnia*. *Memorie dell' Istituto Italiano di Idrobiologia* 45: 245-284.

Zhang L & Baer KN (2000). The influence of feeding, photoperiod and selected solvents on the reproductive strategies of the water flea, *Daphnia magna*. *Environmental Pollution* 110: 425-430.

Capítulo II

Respostas individuais e populacionais de *Daphnia* a variações na quantidade de alimento: a influência do tamanho corporal na eficiência alimentar.

NOTA PRÉVIA: O capítulo II corresponde a um artigo científico que se encontra presentemente submetido para publicação numa revista internacional com arbitragem científica. Encontra-se, portanto, inteiramente escrito em língua inglesa, pelo que previamente se apresenta um resumo traduzido do trabalho constante do capítulo, acompanhado de uma lista de palavras-chave que a ele poderão ser associadas.

Resumo | A dinâmica das comunidades fitoplânctonicas em ecossistemas de água doce constitui um importante condicionante para o zooplâncton, podendo mesmo ser considerada um factor de *stress* natural que pode afectar, eventualmente em apreciável extensão, a cascata trófica. Os zooplanctontes filtradores, dos quais o género *Daphnia* é exemplo incontornável, lidam com as flutuações nos seus recursos alimentares ajustando a sua história de vida de acordo com o equilíbrio dinâmico através do qual se regem as estratégias de alocação da energia disponível em cada momento. Populações naturais de *Daphnia*, que coexistam num mesmo lago, competem pelos recursos existentes (que frequentemente são escassos), sendo que, geralmente, se assume que o tamanho corporal será um parâmetro determinante da capacidade de exploração de recursos apresentada pelos organismos de cada uma destas populações. Neste trabalho foram avaliadas as respostas individuais (parâmetros de história de vida) e populacionais de uma população de *Daphnia magna* e três populações de *Daphnia* c.f. *longispina* ao longo de um gradiente discreto estabelecido com concentrações de alimento (alga verde, *Pseudokirchneriella subcapitata*). Assim, a metodologia experimental utilizada permitiu abordar fundamentalmente duas questões: (1) quais as diferenças entre as estratégias gerais usadas por estas populações para regular a sua história de vida, quando são sujeitas a concentrações baixas, médias e elevadas de alimento; (2) em que medida os organismos de maior tamanho corporal (*D. magna*) são mais eficientes na exploração de recursos do que os de menor tamanho corporal (*D.* c.f. *longispina*). Os resultados obtidos indicam que o tamanho corporal não será o parâmetro mais relevante na determinação da capacidade de exploração de recursos de diferentes populações de *Daphnia*. As populações de *D.* c.f. *longispina* foram frequentemente mais eficientes, tanto a nível individual como populacional, na exploração de diferentes concentrações de alimento, sendo que, quando comparadas entre si, estas populações apresentaram diferenças importantes na capacidade de exploração de um mesmo nível alimentar. Mais ainda, estas diferenças de eficiência observadas foram, em alguns casos, de maior escala do que as que foi possível registar quando se comparou *D. magna* com cada uma das populações de *D.* c.f. *longispina*.

Palavras-chave | *Daphnia magna*; *Daphnia* cf *longispina*; respostas populacionais; *fitness*; capacidade de exploração de recursos alimentares; tamanho corporal.

Daphnia life-history and fitness over a discrete food gradient: is body size the single reliable trait in predicting exploitative ability?

Joana Luísa Pereira & Fernando Gonçalves

CESAM& Departamento de Biologia da Universidade de Aveiro, 3810-193 Aveiro, Portugal.

Abstract | Phytoplankton dynamics in freshwater ecosystems can be faced as a meaningful natural stressor for its main grazers, and hence constrain bottom-up interactions within the trophic structure of the food-web. Filter-feeding zooplankters, such as *Daphnia*, cope with resources fluctuations by adjusting their life-history as a function of balances in the energy allocation rules. Additionally, *Daphnia* populations often withstand interspecific competition within coexisting related species, and it is generally assumed that body size is a strategic trait conditioning exploitative ability and the related competitive advantage of species. The life-history responses of one *Daphnia magna* population and three populations of *Daphnia* cf *longispina* to a discrete gradient of algae concentration were assessed. This experiment was focused on (i) how these distinct populations regulate life-history when feeding over low, intermediate and high food-levels; and on (ii) whether the large-bodied *D. magna* can be better in exploiting resources over the gradient than can the smaller *D.* cf *longispina* populations. We found evidences that body size might not be the single trait influencing the exploitative ability of related species. The *D.* cf *longispina* populations were often better than *D. magna* in exploiting different levels of resources, and remarkable differences in fitness were found between these similar-sized individuals within food-level; indeed, these differences were frequently of higher scale than those found between the larger *D. magna* and any of the *D.* cf *longispina* populations.

Key-words| *Daphnia magna*; *Daphnia* cf *longispina*; life-history; fitness; exploitative ability; body size.

INTRODUCTION

Cladocerans, and specifically those belonging to the genus *Daphnia*, are widely used as model organisms in aquatic ecology, evolutionary biology and ecotoxicology. These organisms are particularly suitable as experimental organisms *i.e.* are easy to rear, have short and productive life-cycles, and are cyclical parthenogens (allowing the control genotypic variance within and between experiments). The relevance of cladocerans as model organisms lies also on the ecological key-role played by these populations in the food web dynamics of the pelagic zone (Benzie 2005): they constitute an important food resource for many fish species and are the main grazers upon the phytoplankton assemblages, therefore assuming a pivotal role in the trophic interactions occurring in freshwater ecosystems (Tessier et al. 2000).

Either externally driven environmental changes or the natural grazing dynamics can affect the phytoplankton composition and/or abundance in lentic water bodies. The way cladoceran populations cope with these fluctuations in resources availability is closely related with their exploitative abilities. *Daphnia* life-history traits such as time to onset reproduction, net fecundity scores, offspring size/quality, growth, and the related population fitness have been shown to be linked to variations in food availability (e.g. Taylor & Gabriel 1985, Lynch 1989, Tessier & Consolatti 1991, Glazier 1992, Guisande & Gliwicz 1992, Taylor & Gabriel 1992, Boersma & Vijverberg 1994, Boersma 1995, Trubetskova & Lampert 1995, Boersma 1997a,b). These reported changes in life-history are likely to be promoted by adjustments in the strategies for the allocation of assimilated energy; i.e. result from the balance of investments in reproduction, growth, and/or maintenance (Kooijman 1986, McCauley et al. 1990, Arendt 1997, Polishchuk & Vijverberg 2005, Rinke & Vijverberg 2005). In short, as resources get depressed, daphnids generally disinvest in reproduction and start giving priority in energy allocation to growth and/or maintenance in order to ensure longevity and lifetime reproduction.

Interspecific competition for resources among closely related species is common within zooplankton communities, and, according to the resource-ratio theory (Tilman 1980) the better competitor within coexisting organisms would be the species able to keep a positive growth rate at lower resource availability. By addressing interspecific competition between ecologically related species (e.g. zooplankton species), the Size Efficiency Hypothesis (Brooks & Dodson 1965) provided the theoretical grounds to explain the dominance of large-bodied over the smaller zooplankton species, in lakes where predation pressure is low or absent: the food concentration need to permit the population maintenance (where population growth equals zero) should decrease as adult body size of coexisting species increases (Brooks & Dodson 1965, Hall et al. 1979). The influence of body size on the grazing activity, and consequently on *Daphnia* fitness, has been significantly explored in literature since then. It was shown that large-bodied *Daphnia* species have lower threshold levels for food resources *i.e.* outcompete coexisting smaller species (*e.g.* Gliwicz 1990, Kreutzer & Lampert 1999); greater efficiency in food collection and lower metabolic costs of the larger of related species would theoretically enable them to accrue a competitive advantage under resources-limited environments (Hall et al. 1979). The relationship between body size and exploitative ability at unlimited food resources has also been studied and the same competitive advantage of larger morphs seems to exist (Tessier & Goulden 1987, Declerck et al. 1997, Tessier et al. 2000). This competitive advantage of the large-bodied of related species is not consensual in the literature, and

experimental evidences of opposite patterns have been reported, either considering food shortage or unlimited food supply (Lynch 1979; Tillmann & Lampert 1984, Stemberger & Gilbert 1985, Tessier & Goulden 1987). Remarkable differences in exploitative ability have also been found between species similar in body size and between distinct genotypes within the same species (e.g. Boersma & Vijverberg 1994, Epp 1996, Tessier et al. 2000, Tessier & Woodruff 2002).

In the present study, we analysed significant life-history endpoints (particularly those related with fecundity, somatic growth and population growth) of four different *Daphnia* populations feeding over a discrete food quantity gradient. Moreover, the life-history performance and fitness of the large-bodied *Daphnia magna* was compared with that of three distinct small-bodied species belonging to the *Daphnia longispina* complex, in order to address the relationship between body size and exploitation efficiency, and concomitantly analyze whether this relationship changes along the food gradient. By comparing life-history and fitness estimates among the similar-sized *D.* cf *longispina* populations, and between these populations and *D. magna*, we intended to access whether the *taxa*-specific plasticity in life-history traits and fitness can balance the advantage of a larger body size in exploiting resources i.e. whether exploitative ability may be constrained by genotype-specific functional diversity rather than singly by size.

MATERIAL & METHODS

Monoclonal bulk cultures of *Daphnia magna* (clone A *sensu* Baird et al. 1989), and of three *Daphnia* cf *longispina* clonal lineages were reared in the lab, under a 16:8 hr light:dark photoperiod, at a temperature of 20±2ºC, in synthetic ASTM hardwater medium (ASTM 1980) supplied with an organic additive (5ml/L) extracted from the algae *Ascophyllum nodosum* (Baird et al. 1989). Cultures were renewed every other day and the organisms were fed with *Pseudokirchneriella subcapitata* (Korshikov) Hindak [cyclically cultured in lab in Woods Hole MBL medium (Stein 1973) under a 24:0 hr light:dark cycle with a 20±2ºC room temperature]. The three *D.* c.f. *longispina* clonal lineages used in tests were established from field-collected samples: (1) clone M was collected in lake Mira (Mira, centre-northwest of Portugal) and has been maintained in the lab since 2001 (clone EM7 *sensu* Antunes et al. 2003); (2) clone V resulted from a sample picked in lake Vela (Quiaios, centre-northwest of Portugal) in 2004; (3) clone T was collected in the shallow reservoir Tapada Grande (Mértola, southeast of Portugal) in 2004. The species belonging to the subgenus *Hyalodaphnia* (commonly called *Daphnia longispina* group) are genetically well differentiated, however interspecific

hybridisation and backcrossing within the group often occurs (Schwenk & Spaak 1995, Schwenk et al. 2000). High rates of interspecific hybridization, and a high degree of phenotypic plasticity observed in some traits constrain appreciably a morphotype-based classification of the species/*taxa* within *Hyalodaphnia* (Schwenk et al. 2000, Billiones et al. 2004). ITS-RFLP techniques (Billiones et al. 2004) confirmed that our clones represent three distinct *taxa* within the *D. longispina* complex (Petrusek et al. 2005): although all the populations morphologically resemble *D. longispina* (according to Benzie 2005), the genotypes M, V and T are consistent with ITS-RFLP patterns and 12S DNA sequences of *D. galeata* x *longispina*, *D. longispina* x *galeata* and *D. galeata*, respectively. Hereinafter, we will refer to the different genotypes as *D. longispina* M, V and T for text clarity convenience.

The effects of different food concentrations in the life-history of the four *Daphnia* genotypes (*D. magna* and *D. longispina* M, V and T) were analysed. The life-history endpoints were assessed after a 21-day exposure of the organisms to several food-levels; in order to standardize procedures and the test design, we generally followed the OECD guideline for reproduction bioassays with *Daphnia* (OECD 1998). The animals were held during 21 days in 50ml glass beakers filled with ASTM hardwater, supplied with the *A. nodosum* additive. Renewal occurred every other day and the organisms were fed daily according with the food treatment ranges. Five food-levels were used as treatments in each experiment: 0.0, $0.375x10^5$, $0.75x10^5$, $1.5x10^5$, $3.0x10^5$cells/ml of *P. subcapitata*. Ten replicates, with a single individual each, were used as replicates within each treatment. All the tests started with newborns ageing less than 24-hrs, born in the bulk cultures between the 3rd and the 5th brood, in order to minimise maternal effects (Barata and Baird, 1998).

The tests were screened daily for eventual mortality and for progeny-related records. When present, offspring were counted and immediately removed. The neonates yielded in the first brood were measured (five individuals per female were used as sample). The body size of the females was estimated immediately after the release of the first brood, and at the beginning and at the end of the test, by extrapolation from the moult exopodite length (Pereira et al. 2004), allowing the calculation of the somatic growth rate (SGR) through the following equation:

$SGR = [\ln(l_f) - \ln(l_0)] / \Delta t$ (day^{-1}), where l_f is final body length (mm), l_0 is initial body length (mm) and Δt is time range (days). All measurements were made under stereoscope (Olympus SZX9).

Fecundity- and survival-related data were integrated for the estimation of the *per capita* rate of population increase (r; day^{-1}) through the Euler- Lotka equation:

$1 = \sum_{i=1}^{n} e^{rx} l_x m_x$, where x stands for age class (days), l_x for probability of surviving to age x, and m_x for fecundity at age x. Uncertainties were estimated following the Jackknife technique (Meyer et al. 1986).

Data analysis

One-Way Analysis of Variance (ANOVA) was used to compare the life-history responses of each *Daphnia* population to increasing food-levels (four algae concentrations: 0.375×10^5 – 3.0×10^5 cells/ml). When applicable, the post-hoc Tukey HSD test was used in order to properly assign statistically different food-treatments (Quinn & Keough 2002). Given the species-specific differences between *D. magna* and *D.* cf *longispina* in life-history and our aim of comparing all the *taxa* in between, we focused on whether there are relative changes in the endpoints/parameters tested as food quantity varies. The data set was therefore transformed by relating each record to the highest record obtained within species/*taxa* in each endpoint of interest, considering the four higher food treatments tested. The appropriate arcsine transformation was then applied prior to any statistical procedure (Quinn & Keough 2002). A two-way ANOVA was used to assess the significance of the effects of food quantity and *taxa*, as well as of their interaction, on the life-history endpoints and on the population growth rate. The strength of association of each factor and interaction to the ANOVA model was addressed through the calculation of the partial Eta-squared parameter. As highly significant interactions were consistently detected, a one-way ANOVA was used within each food-level, to test whether *taxa* differ from each other depending on the food availability; and when applicable, the Tukey HSD *post-hoc* test was carried out in order to assign statistically significant differences between *taxa* within food-level (Quinn & Keough 2002). All statistical procedures were carried out under significance level (α) of 0.05.

RESULTS

If one excludes the treatment where no food was added, mortality was rarely observed in the tests (10% for *D. magna*, 2.5% for *D. longispina* M and 5% for *D. longispina* V and T), and could

never be related with the test treatments. In the treatment where no food (i.e. algae) was added, the proper analysis of the life-history parameters was severely constrained: despite the maintenance of the organic supplement (see "Materials & Methods") along the 21 days of the tests, mortality was high (20-40% depending on the *Daphnia* population) and the surviving animals were often unable to reproduce, which compromises the statistical analysis of several endpoints. Therefore, data relative to this treatment are occasionally mentioned in the text and are shown in the figures for providing merely indicative information on the effects of starvation, but were not considered for statistics.

As a response to an increasing gradient of food concentration all the tested *taxa* showed a general amelioration of the overall life-history performances. As the food-level raised the organisms yielded significantly larger offspring broods, often reproduced earlier (FIGURE II.1 - a and b), and generally recorded significantly higher population growth rates, *r* (FIGURE II.2 – b; TABLE II.I). However, *D. longispina* V was found to be an exception: although net fecundity was significantly affected along the food gradient, no significant differences in *r* were detected between food treatments. Actually, this was the least responsive population to changes in food ration, followed by *D. longispina* T (FIGURE II.1 – a and b; TABLE II.I): *D. longispina* V showed large tolerance to food shortage i.e. only net fecundity (number of neonates yielded per female along the 21 days of the test) suffered a significant impairment as food decreased (TABLE II.I); *D. longispina* T fecundity and *r* were significantly impaired whereas none of the other analysed endpoints registered significant changes due to variation in food quantity. Both the fecundity endpoints and the growth rates of *D. magna* suffered changes along the food gradient (FIGURE II.1 – a-d; FIGURE II.2 – a, b), which were often statistically significant (TABLE II.I). It seems that this population was the most sensitive to the decrease of food availability, but similarly to *D*. cf *longispina* populations, net fecundity and *r* were more responsive than any of the other analysed endpoints (all food treatments promoted significantly different responses in both endpoints – TABLE II.I). As food increases, *D. magna* grew faster and followed a consistent pattern in reproducing earlier, although at a slightly smaller size and having slightly smaller neonates (FIGURE II.1 b-d, FIGURE II.2 a; TABLE II.I). Endpoints such as age at first reproduction (AFR), size of N1 neonates, and primipara size, were indeed found to be less responsive particularly when considering the *D. longispina* populations (TABLE II.I). *D. longispina* M was the single population anticipating significantly the release of a first brood with smaller neonates as food ration raises.

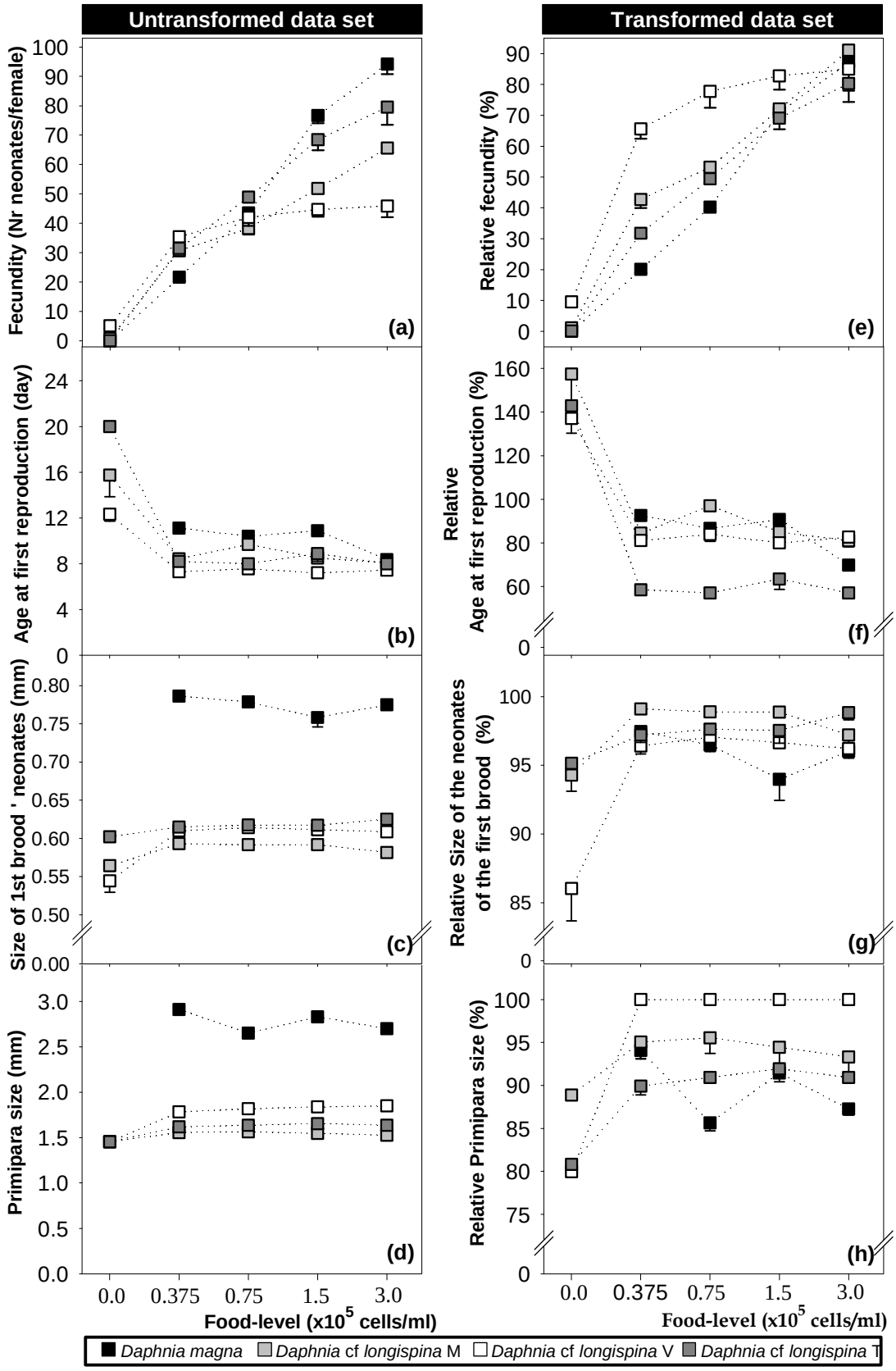


FIGURE II.1 | Life-history responses of *Daphnia* spp. over a discrete gradient of food concentration: a-d – direct plots obtained from the actual records on the life-history endpoints; e-f – plots regarding the relative changes in the endpoints along the food gradient. Error bars represent the standard error.

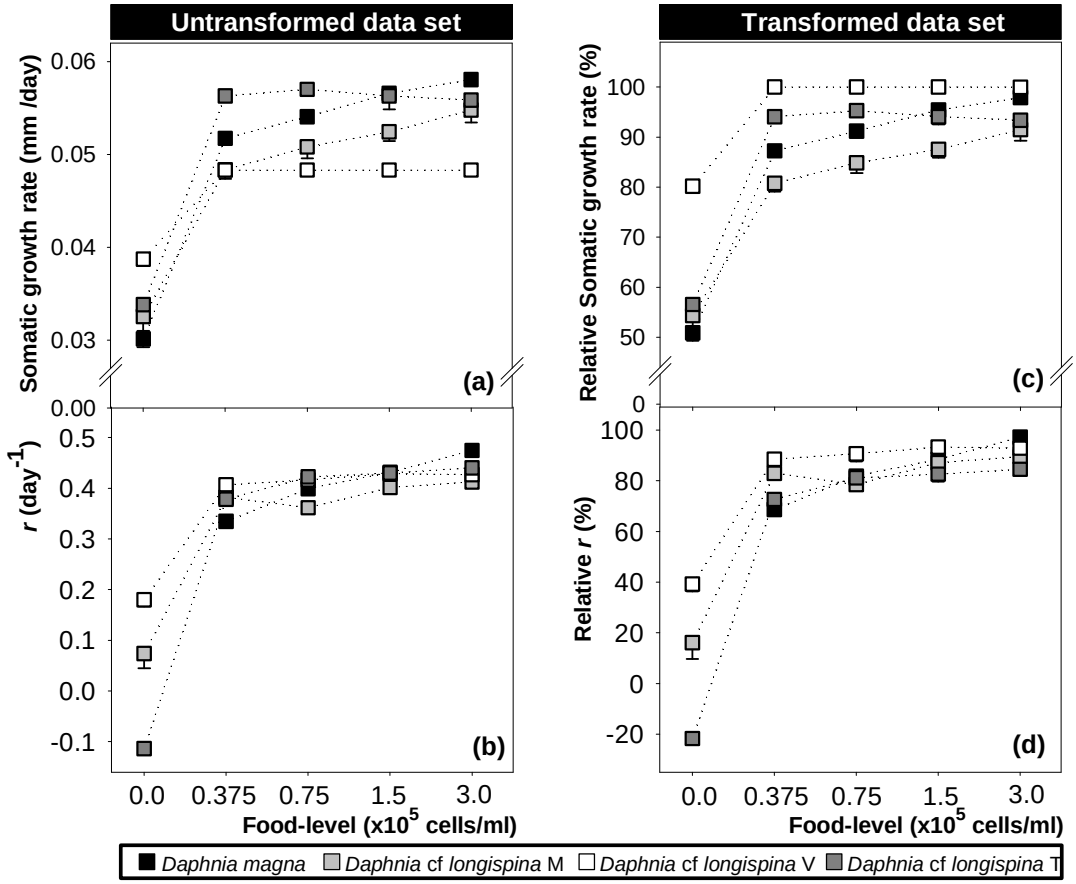


FIGURE II.2 | Somatic- and population growth rates exhibited by the *Daphnia* populations in response to a discrete food gradient; a, b – direct plots obtained from the actual records made during the experiments; c-d - plots regarding the relative changes in the growth parameters along the food gradient. Error bars represent the standard error.

Under near-starvation conditions (no food added), *D. magna* showed a very low somatic growth rate (SGR) and was unable to reproduce at all, which compromised the calculation of the respective *r* (FIGURE II.1-a; FIGURE II.2-a, b). In addition, very little offspring was yielded by any of the *D. longispina* populations (average fecundity: 0.8, 5.2 and 0.1 neonates for *D. longispina* M, V and T, respectively). The concentration-response curves relative to all the remaining endpoints/parameters consistently denote very serious impairment in life-history of all starving *D.* cf *longispina* populations: slightly smaller females released a first brood of slightly smaller neonates, with a considerable delay when comparing this with any of the other food treatments (FIGURE II.1 – b-d); somatic growth rates were recorded below 0.04, and population growth rates were found either negative (*D. longispina* T) or below 0.2 day^{-1} (FIGURE II.2 – a, b).

TABLE II.I: One-Way ANOVA summaries relative to the life-history endpoints analysed within each *Daphnia* population (df – degrees of freedom; MS_{res} – residual Mean Squares; AFR – Age at First Reproduction; SGR – Somatic Growth Rate). Untransformed data were used in this analysis in order to compare food treatments within each *Daphnia* population. When available, statistically significant differences between food treatments (Tukey test, $P<0.05$) are also assigned in the table, using the letters a-d.

	Endpoint	df	MS_{res}	*F* ratio	*P* value	Tukey test [food-levels (cells/ml)]			
						$0.375x10^5$	$0.75x10^5$	$1.5x10^5$	$3.0x10^5$
D. magna	Fecundity	3, 32	44.125	208.699	<0.001	a	b	c	d
	AFR	3, 32	0.252	51.258	<0.001	a	a	a	b
	Sz N1 neonates	3, 32	$4.23\ e^{-4}$	2.998	0.045	a	ab	b	ab
	Primipara size	3, 32	0.0078	16.812	<0.001	a	b	a	b
	SGR	3, 32	$7.81\ e^{-6}$	8.632	<0.001	a	ab	bc	c
	r	3, 36	$3.20\ e^{-4}$	108.118	<0.001	a	b	c	d
***D. longispina* M**	Fecundity	3, 35	32.542	69.910	<0.001	a	b	c	d
	AFR	3, 35	0.849	5.725	0.003	b	a	c	c
	Sz N1 neonates	3, 35	$5.45\ e^{-5}$	5.054	0.005	a	a	a	b
	Primipara size	3, 35	0.0090	0.271	0.846	--	--	--	--
	SGR	3, 35	$1.30\ e^{-5}$	5.362	0.004	a	ab	ab	b
	r	3, 36	$7.74\ e^{-4}$	6.453	0.001	b	a	b	b
***D. longispina* V**	Fecundity	3, 34	70.335	3.053	<0.001	a	ab	ab	b
	AFR	3, 34	0.357	0.651	0.588	--	--	--	--
	Sz N1 neonates	3, 34	$1.01\ e^{-4}$	0.472	0.704	--	--	--	--
	Primipara size	3, 34	0.0034	2.407	0.084	--	--	--	--
	SGR	3, 34	$3.04\ e^{-11}$	1.081	0.370	--	--	--	--
	r	3, 36	$7.59\ e^{-4}$	1.325	0.281	--	--	--	--
***D. longispina* T**	Fecundity	3, 34	112.925	39.237	<0.001	a	b	c	c
	AFR	3, 34	1.073	1.516	0.228	--	--	--	--
	Sz N1 neonates	3, 34	$1.44\ e^{-4}$	1.300	0.290	--	--	--	--
	Primipara size	3, 34	0.0044	1.318	0.285	--	--	--	--
	SGR	3, 34	$9.69\ e^{-7}$	2.180	0.108	--	--	--	--
	r	3, 36	$8.14\ e^{-4}$	9.080	<0.001	a	b	b	b

Regarding food-level and species/*taxa* as contributing factors to the overall effects observed in life-history endpoints, statistics consistently found highly significant interactions; the single exception to this statistical output was assigned to primipara size, where "*Taxa*" seems to be the single factor independently contributing to the overall variation (TABLE II.II). In fact, the relative spatial order of *taxa* changes in the plots as food concentration varies (FIGURE II.1 [e, g, h]; FIGURE II.2 [c,d]), which graphically resembles the statistically determined interaction (see e.g. *D. magna* fecundity as a clear picture of this graphical feature). The estimation of the strength of association (partial Eta-squared – TABLE II.II) depicted "Food" as the factor contributing more for the registered effects in Fecundity and *r*. "*Taxa*" had a higher contribution in explaining the variance of the remaining life-history endpoints

i.e. AFR and primipara size - where no significant effect of "Food" was actually noticed –, size of N1 neonates and SGR.

TABLE II.II: Two-Way ANOVA summary relative to the life-history responses of *Daphnia* feeding on an increasing gradient of food quantity (df – degrees of freedom; MS – Mean Squares). The data set was normalised *a priori* by using the highest record obtained in each endpoint within each population, and the treatment were no food was added was excluded from the analysis. Partial Eta-squared (Partial η^2) values were added to the table as a measure of the contribution by each factor and interaction to the overall effects.

Endpoint	Source of variation	df	MS	*F* ratio	*P* value	Partial η^2
Fecundity	Food	3, 135	2.14173	97.22	0.000	0.684
	Taxa	3, 135	0.56486	25.64	0.000	0.363
	Food x *Taxa*	9, 135	0.08416	3.82	0.000	0.203
Age at first reproduction	Food	3, 135	0.14570	8.42	0.000	0.158
	Taxa	3, 135	1.02778	59.38	0.000	0.569
	Food x *Taxa*	9, 135	0.09678	5.59	0.000	0.272
Size of N1 neonates	Food	3, 135	0.00175	0.40	0.757	0.009
	Taxa	3, 135	0.07144	16.16	0.000	0.264
	Food x *Taxa*	9, 135	0.01208	2.73	0.006	0.154
Primipara size	Food	3, 135	0.01029	0.914	0.436	0.020
	Taxa	3, 135	0.18946	16.83	0.000	0.272
	Food x *Taxa*	9, 135	0.01981	1.76	0.082	0.105
Somatic Growth Rate	Food	3, 135	0.08809	13.65	0.000	0.233
	Taxa	3, 135	0.86431	133.89	0.000	0.748
	Food x *Taxa*	9, 135	0.04317	6.69	0.000	0.308
r	Food	3, 144	0.28487	33.16	0.000	0.409
	Taxa	3, 144	0.19995	23.28	0.000	0.327
	Food x *Taxa*	9, 144	0.05731	6.67	0.000	0.294

TABLE II.III: One-Way ANOVA summaries relative to the life-history endpoints analysed within each food-level (df – degrees of freedom; MS_{res} – residual Mean Squares; AFR – Age at First Reproduction; SGR – Somatic Growth Rate). Data normalisation was undertaken prior to analysis, as specified in Table II, and the *post-hoc* Tukey test was used, when applicable, to assign differences between *taxa* responding to each food ration (letters a-d in the table).

Food Treatment	Endpoint	df	MS_{res}	*F* ratio	*P* value	Tukey test (*Taxa*)			
						D m	*Dl* M	*Dl* V	*Dl* T
0.375x10^5 cells/ml	Fecundity	3, 34	0.00556	72.373	<0.001	b	d	a	c
	AFR	3, 34	0.0142	24.796	<0.001	a	ab	b	c
	Sz N1 neonates	3, 34	0.00302	7.718	<0.001	b	a	b	b
	Primipara size	3, 34	0.0110	6.185	0.002	ab	a	b	b
	SGR	3, 34	0.00121	305.921	<0.001	c	d	a	b
	r	3, 36	0.00671	20.788	<0.001	b	a	a	b
0.75x10^5 cells/ml	Fecundity	3, 35	0.0156	20.728	<0.001	b	b	a	b
	AFR	3, 35	0.0177	36.180	<0.001	b	a	b	c
	Sz N1 neonates	3, 35	0.00387	4.526	0.009	b	a	a	a
	Primipara size	3, 35	0.0084	13.248	<0.001	b	a	b	b
	SGR	3, 35	0.00499	53.469	<0.001	c	d	a	b
	r	3, 36	0.00686	11.620	<0.001	b	b	a	b

1.5x10^5 cells/ml	Fecundity	3, 34	0.0189	4.327	0.011	b	b	a	b
	AFR	3, 34	0.0270	6.514	0.001	a	a	ab	b
	Sz N1 neonates	3, 34	0.00712	4.947	0.006	b	a	a	a
	Primipara size	3, 34	0.0142	2.136	0.114	--	--	--	--
	SGR	3, 34	0.00797	27.424	<0.001	b	c	a	bc
	r	3, 36	0.0132	2.876	0.049	ab	ab	a	b
3.0x10^5 cells/ml	Fecundity	3, 32	0.0498	0.600	0.620	--	--	--	--
	AFR	3, 32	0.00983	18.094	<0.001	b	a	a	c
	Sz N1 neonates	3, 32	0.00365	8.257	<0.001	b	b	b	a
	Primipara size	3, 32	0.0115	3.566	0.025	b	a	ab	ab
	SGR	3, 32	0.0120	11.939	<0.001	a	b	a	b
	r	3, 36	0.00763	15.045	<0.001	a	bc	ab	c

When compared to the *D.* cf *longispina* populations and despite presenting better overall absolute records in life-history endpoints (FIGURE II.1 – a-d; FIGURE II.2 – a, b), the larger *D. magna* was never clearly superior in adjusting to the food constraints. Moreover, the differences between *D.* cf *longispina* populations were often of higher magnitude than those between the large-bodied *D. magna* and any of the small-bodied *D.* cf *longispina* (see e.g. FIGURE II.1 e – 0.375x10^{5}cells/ml). When regarding the lowest food-level, *D. magna* was the population yielding the lowest relative number of neonates along the test, and delaying more the release of the first brood (FIGURE II.1 e, f). *D.* cf *longispina* populations feeding in this food-level also differed markedly in between when regarding relative fecundity and age at first reproduction: *D. longispina* V showed the highest relative fecundity along the test, and *D. longispina* T reproduced earlier but recording the lowest relative offspring yield. Statistics was strictly consistent with the observed patterns (TABLE II.III). *D. longispina* V started reproduction at the largest relative size, followed by *D. magna* and *D. longispina* M that actually did not statistically differ when responding to low food availability; no statistically significant differences were found between the size of the neonates from the first brood released by *D. magna*, *D. longispina* V and *D. longispina* T (FIGURE II.1 g, h; TABLE II.III). As food availability increases these patterns change, and a trend to the smoothing of differences between populations should be noticed (TABLE II.III; FIGURE II.1 – e-h). Although *D. magna* and *D. longispina* T reproduced significantly earlier, the relative fecundity of all populations was statistically similar in the highest food-level; *D. longispina* T was the single population yielding significantly larger neonates in the first brood under the highest food ration, despite slight differences were found between the primipara sizes of the four populations.

When globally comparing calculated growth rates (SGR and *r*) with the remaining life-history endpoints, differences between populations along the entire food gradient seem graphically less marked. The above mentioned trend for a slight smoothing of the graphical distance between populations in the plots as food raises was kept when regarding the somatic growth rate (FIGURE II.2c): while at $0.375x10^{-5}$ cells ml^{-1} all populations showed a statistically different somatic growth, at the highest food supply *D. magna* was statistically similar to *D. longispina* V and *D. longispina* M did not statistically differed from D. longispina T (TABLE II.III). Regardless the food-level, *D. longispina* V was always the population keeping the highest relative somatic growth records while *D. longispina* M showed always the slower relative somatic growth rate (FIGURE II.2c). *D. magna* was the population having the most severe changes in fitness (i.e. population growth rate, *r*) along the food gradient; whereas *D. longispina* T and *D. longispina* V generally kept the lower and the higher (respectively) fitness values along the food gradient (including in the treatment without food supply), *D. magna* was not able to sustain the best relative fitness shown at the highest food level when food supply decreases (FIGURE 2d; TABLE III).

DISCUSSION

Variations on the availability of food can constitute a relevant environmental stressor of natural communities, representing a direct constrain on the energy uptake and consequently on the energy status of an organism. The dynamics of phytoplankton communities in lentic ecosystems leads to natural fluctuations in resources availability for grazer zooplankters such as *Daphnia*. Daphnids are known to deal with these resources variation by changing their life-history performances to optimally allocate the available resources (Guisande & Gliwicz 1992, Boersma 1997a). Several authors have already stressed that food availability is of main importance for *Daphnia* reproduction, growth and ultimately survival (e.g. Guisande & Gliwicz 1992, Boersma 1995, Trubetskova & Lampert 1995, Epp 1996, Antunes et al. 2003, Polishchuk & Vijverberg 2005).

Energy and nutrients must be budgeted among different functions in an organism, and resources availability will condition the allocation to a given function often at expenses of a disinvestment in another. The models of energy allocation in starving daphnids by Kooijman (1986) and McCauley et al. (1990), although differing in their mechanistic grounds, assume that maintenance, rather than growth or reproduction, should be the main priority regarding longevity and lifetime reproductive potential. Indeed, experimental evidence supports these models showing that

under high food availability daphnids commit a great part of their energy budget to boost reproduction; as food resources depress disinvestment in reproduction and then in growth occurs, and thus smaller females release smaller broods of often larger, e.g. more hunger-resistant neonates (Tessier & Consolatti 1991, Glazier 1992, Gliwicz & Guisande 1992, Taylor & Gabriel 1992, Trubetskova & Lampert 1995, Boersma 1997a, Polishchuk & Vijverberg 2005). Our results were generally consistent with these energy allocation models/patterns. All *taxa* showed better reproductive performances as food raised. Along with food shortage the *Daphnia* populations would first disinvest in reproduction and would try to keep growth, although at slower rates. Starving *Daphnia* (no food supply) showed very poor offspring production and growth rates, which provides a clear picture of the extreme situation where maintenance of body condition becomes single priority.

Tessier & Consolatti (1991) found that *Daphnia* are able to adapt mean neonate size in response to food density, whereas Glazier (1992) proposed a model attempting to overcame existing experimental controversy on the relationship between egg/offspring size and food availability. When no food was added the *D. longispina* populations (*D. magna* was not able to reproduce at all) seem to respond in agreement with this latter model by producing very small offspring due to a reproductive constraint; when food was added, however, only week inferences could be made on the fit of our experimental results to either model since the size of the neonates released in the first brood, as well as primipara size, rarely changed significantly along the food gradient. Indeed, the relationship between food availability and egg/neonate size and/or quality is not unequivocal nor consistent with theoretical predictions: egg mass was already found to be similar in *Daphnia* raised at different food rations (Taylor & Gabriel 1985), and despite not finding differences in size between offspring born under different food-levels, Tessier & Consolatti (1991) reported higher quality offspring at low food-levels; Boersma (1995) reported smaller but higher-quality neonates produced by *D. galeata* feeding on low food-levels, but this pattern was not confirmed by the author with *D. magna* (Boersma 1997b). Our results convey no patterns for remarkable variation in offspring size as a function of food concentration (at least under the tested food range). Similarly, primipara did not differ expressively in size along the food gradient which is in agreement with previous studies (e.g., Kooijman 1986, Lynch 1989, Rinke & Vijverberg 2005). This independence of primipara body size on food availability is attributed to the existence of a critical size for the onset of reproductive investment (Lynch 1989) and hence food concentration would rather influence the time need to reach that size.

The intrinsic rate of population increase (*r*) integrates reproductive endpoints and survival, and can therefore be faced as a measure of the populations' fitness (Kammenga et al. 1996); it provides a more feasible estimate of the general ecological impacts of environmental changes in natural populations, such as fluctuating food resources, than do the individual-level life-history endpoints (Kammenga et al. 1996, Forbes & Calow 1999). The fitness of the *Daphnia* populations was responsive to changes in food availability. When facing food shortage, daphnids generally lowered their population growth rates, which is a documented pattern in several organisms and it is likely to be a consequence of an effort to maximise the efficiency of resources use [see the review by Arendt (1997)]. Given the residual mortality observed in our study, the main contributions for the population growth rates would be the fecundity rates and the time that daphnids take to release the first brood (Meyer et al. 1986; Vanni & Lampert 1992). Changes in these reproductive endpoints with food-level, due to shifting and/or adjustment in energy allocation strategies (see above) seem though to be of importance on population fitness.

The relationship between exploitative ability of cladoceran species and body size has been faced as evidence of the Size Efficiency Hypothesis postulates (Brooks & Dodson 1965, Hall et al. 1976): when competing for food resources, small-bodied daphnids would be eliminated by the larger forms, which should be related to a more efficient food collection and a relatively reduced metabolic demand per unit of body mass of the latter. Evidences exist of the advantage of large-bodied cladocerans in exploiting low-food conditions i.e. of the increase of food threshold concentration with body size (Gliwicz 1990, Kreutzer & Lampert 1999). Competitive advantage of larger morphs of similar species feeding in unlimited/high-level food resources was also documented (Tillmann & Lampert 1984, Tessier & Goulden 1987, Declerck et al. 1997, Tessier et al. 2000, Tessier et al. 2001) and seems to be related with their higher filtering rates and broad food particle size range (e.g. Declerck et al. 1997, Tessier et al. 2001). Our results were not generally consistent with these trends: when comparing *D. magna* with the *D. longispina* populations within food-level, the large-bodied species was rarely found to be the more effective species in taking advantage of the food resources; and, *D. magna* only showed the best records when considering the highest food-level and only for growth rates (somatic growth rates and *r*) i.e. the small-bodied *Daphnia* c.f *longispina* populations were often better in exploiting food resources regardless its level of availability or the endpoint of interest.

In fact, there is some experimental controversy regarding the insight on exploitative competition by the Size Efficiency Hypothesis. In a field study, Lynch (1979) has indeed registered the superiority of smaller species in lakes where size-specific predation was absent. In their experiments with differently-sized daphnids, Tillmann & Lampert (1984) found that, despite inferior under unlimited resources, the smaller organisms were better competitors given they were able to reproduce even when facing food scarcity. Tessier & Goulden (1987) recorded the specific juvenile growth and showed that, under low food-levels, small body size seems advantageous. Important differences in exploitative ability were also already evidenced, either considering similar-sized species (Boersma & Vijverberg 1994, Tessier & Woodruff 2002) or even different genotypes of the same species (e.g., Epp 1996, Tessier et al. 2000, Antunes et al. 2003). Considering our specific experimental design, *Daphnia* species followed these patterns rather than those predicted by the Size Efficiency Hypothesis. The smaller *D. longispina* showed better reproductive performance and fitness than *D. magna*, along the food gradient, denoting that there is no apparent relationship between body size and sensitivity to food availability and/or exploitative ability. This can be related with differential plasticity in life-history traits (Tessier & Woodruff 2002) and/or rely on a number of physiological features of smaller species that can compensate for body size: (i) higher specific rate of food collection and higher feeding efficiency that are likely to compensate for greater metabolic requirements (DeMott 1982, Stemberger & Gilbert 1985); (ii) better swimming abilities on a mass-specific basis that can enhance food collection efficiency (Stemberger & Gilbert 1985); (iii) higher efficiency in energy allocation strategies in face of food gradients (Tessier et al. 1983, Tillmann & Lampert 1984, Tessier & Consolatti 1991).

On the other hand, remarkable differences in fitness and life-history within food-level were found between the closely-related and similarly-sized *D. longispina* populations; these differences were frequently higher than those between the larger *D. magna* and each of the *D. longispina* populations. This provides evidence that exploitative ability, and eventually competitive ability, may be driven by genotype specificity rather than have a straight relationship with body size. Assuming *r* as an efficient measure of the populations' fitness (Kammenga et al. 1996) *D. longispina* T and V should be noted as the populations generally withstanding worse and better, respectively, the changes in food availability. In fact, either *D. longispina* M and V are interspecific hybrids between species belonging *D. longispina* complex (see "Material & Methods" for details). The temporal superiority of hybrid genotypes relatively to that of parental species seems to be common and a frequently prevalent phenomenon within *Daphnia* assemblages where both coexist (Schwenk &

Spaak 1995; Temporal Hybrid Superiority Hypothesis). Here, it seems that additional evidence was generated on the apparent exploitative superiority of hybrid genotypes upon the parental species along a gradient of food concentration.

In summary, this study apparently contradicts one of the assumptions of the Size Efficiency Hypothesis by recognising substantial variation in life-history responses and fitness of different *Daphnia* species/*taxa* to food availability, regardless differences or similarities in body size. Body size should not be the single trait conditioning exploitative ability within cladoceran communities. Functional diversity in species responses to changes in resource availability seems to result from evolutionary feedbacks to ecological demands; selection will favour genotypes that can better cope with changing environmental conditions (food concentration) by having e.g. more efficient energy allocation strategies that actually can compensate for body size advantages. Functional traits should hence be additionally considered when modelling interspecific competition and community structure of *Daphnia* assemblages.

REFERENCES

Antunes SC, Castro BB & Gonçalves F (2003). Chronic responses of different clones of *Daphnia longispina* (field an ephippia) to different food-levels. *Acta Oecologica* 24: S325-S332.

Arendt JD (1997). Adaptive intrinsic growth rates: an integration across taxa. *Quaterly Review of Biology* 72: 149-177.

ASTM (1980). Standard practice for conducting acute toxicity tests with fishes, macroinverte- brates and amphibians. Report E 729-80. American Society for Testing & Materials, Philadelphia.

Baird DJ, Soares AMVM, Girling A, Barber I, Bradley MC & Calow P (1989). The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. *In*: H Lokke, H Tyle & F Bro-Rasmussen (Eds.). Proceedings of the First European Conference on Ecotoxicology. Lyngby, pp. 144-148.

Barata C & Baird DJ (1998). Phenotypic plasticity and constancy of life-history traits in laboratory clones of *Daphnia magna* Straus: effects of neonatal length. *Functional Ecology* 12: 442-452.

Benzie JAH (2005). The genus *Daphnia* (including Daphniopsis). Guides to the identification of the Microinvertebrates of the Continental Water of the World – Vol 21. Kenobi productions, Ghent & Backhuys Publishers, Leiden.

Billiones R, Brehm M, Klee J & Schwenk K (2004). Genetic identification of *Hyalodaphnia* species. *Hydrobiologia* 526: 43-53.

Boersma M & Vijverberg J (1994). Resource depression in *Daphnia galeata*, *Daphnia cucullata* and their interspecific hybrid: life-history consequences. *Journal of Plankton Research* 16: 1741-1758.

Boersma M (1995). The allocation of resources to reproduction in *Daphnia galeata*: against the odds? *Ecology* 76: 1251-1261.

Boersma M (1997a). Offspring size and parental fitness in *Daphnia magna*. *Evolutionary Ecology* 11: 439-450.

Boersma M (1997b). Offspring size in *Daphnia*: does it pay to be overweight? *Hydrobiologia* 360: 79-88.

Brooks JL & Dodson SI (1965). Predation, body size, and composition of plankton. *Science* 150: 28-35.

Declerck S, De Meester L, Podoor R & Conde-Porcuna JM (1997). The relevance of size-efficiency to biomanipulation theory: a field test under hypertrophic conditions. *Hydrobiologia* 360: 265-275.

DeMott WR (1982). Feeding selectivities and relative ingestion rates of *Daphnia* and Bosmina. *Limnology and Oceanography* 27: 518-527.

Epp GT (1996). Clonal variation in the survival and reproduction of *Daphnia pulicaria* under low-food stress. *Freshwater Biology* 35: 1-10.

Forbes VE & Calow P (1999). Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? *Environmental Toxicology and Chemistry* 18: 1544-1556.

Glazier DS (1992). Effects of food, genotype, and maternal size and age on offspring investment in *Daphnia magna*. *Ecology* **73**:910-926.

Gliwicz ZM (1990). Food thresholds and body size in cladocerans. *Nature* 343: 638-640.

Gliwicz ZM & Guisande C (1992). Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia* 91: 463-467.

Guisande C & Gliwicz ZM (1992). Egg size and clutch size in two *Daphnia* species grown at different food levels. *Journal of Plankton Research* 14: 997-1007.

Hall DJ, Threlkeld ST, Burns CW & Crowley PH (1976). The size-efficiency hypothesis and the size structure of zooplankton communities. *Annual Review of Ecology and Systematics* 7: 177-208.

Kammenga JE, Busschers M, Van Straalen NM, Jepson PC & Bakker J (1996). Stress induced fitness reduction is not determined by the most sensitive life-history trait. *Functional Ecology* 10: 106-111.

Kooijman SALM (1986). Population dynamics on the basis of budgets. *In*: JAJ Metz. & O Diekman (eds.), The dynamics of physiologically structured populations. Springer Verlag, Berlin, pp. 266-297.

Kreutzer C & Lampert W (1999). Exploitative competition in differently sized *Daphnia* species: a mechanistic explanation. *Ecology* 80: 2348-2357.

Lampert W (2006). *Daphnia*: model herbivore, predator and prey. *Polish Journal of Ecology* 54: 607-620.

Lynch M (1979). Predation, competition, and zooplankton community structure: an experimental study. *Limnology and Oceanography* 24: 253-272.

Lynch M (1989). The life-history consequences of resource depression in *Daphnia pulex*. *Ecology* 70: 246-256.

McCauley E, Murdoch WW, Nisbet RM & Gurney WSC (1990). The physiological ecology of *Daphnia*: development of a model of growth and reproduction. *Ecology* 71: 703-715.

Meyer JS, Ingersoll CG, McDonald LL & Boyce MS (1986). Estimating uncertainty in population growth rates: Jackknife vs. Bootstrap techniques. *Ecology* 67: 1156-1166.

OECD (1998). *Daphnia magna* reproduction test - Test guideline 211. Organization for the Economic Cooperation and Development. Paris.

Pereira JL, Marques CR & Gonçalves F (2004). Allometric relations for *Ceriodaphnia* spp. and *Daphnia* spp. *Annales de Limnogie – International Journal of Limnologie* 40: 11-14.

Petrusek A, Bastiansen F & Schwenk K (2005). European *Daphnia* Species (EDS) - Taxonomic and genetic keys. [Build 2006-01-12 beta]. CD-ROM, distributed by the authors. Dept. Ecology and Evolution, J.W. Goethe-University, Frankfurt am Main & Dept. Ecology, Charles University, Prague.

Pierce CA, Block CA & Aguinis H (2004). Cautionary note on reporting Eta-squared values from multifactor ANOVA designs. *Educational and Psychological Measurement* 64: 916-924.

Polishchuk LV, Vijverberg J (2005). Contribution analysis of body mass dynamics in *Daphnia*. *Oecologia* 144: 268-277.

Quinn GP & Keough MJ (2002). Experimental design and data analysis for biologists. Cambridge University Press, Cambridge. 537p.

Rinke K & Vijverberg J (2005). A model approach to evaluate the effect of temperature and food concentration on individual life-history and population dynamics of *Daphnia*. *Ecological Modelling* 186: 326-344.

Schwenk K & Spaak P (1995). Evolutionary and ecological consequences of interspecific hybridization in cladocerans. *Experientia* 51: 465-481.

Schwenk K, Posada D & Hebert PDN (2000). Molecular systematics of European *Hyalodaphnia*: the role of contemporary hybridization in ancient species. *Proceedings of the Royal Society of London B* 267: 1833-1842.

Stein JR (1973). Handbook of Phycological methods – Culture methods and growth measurements. Cambridge University Press, Cambridge. 448p.

Stemberger RS & Gilbert JJ (1985). Body size, food concentration and population growth in planktonic rotifers. *Ecology* 66: 1151-1159.

Taylor BE & Gabriel W (1985). Reproductive strategies of two similar *Daphnia* species. *Internationale Vereinigung fur theorische und angewandte Limnologie, Verhandlungen* 22: 3047-3050.

Taylor BE & Gabriel W (1992). To grow or not to grow: optimal resource allocation for *Daphnia*. *American Naturalist* 139: 248-266.

Tessier AJ, Henry LL, Goulden CE & Durand MW (1983). Starvation in *Daphnia*: energy reserves and reproductive allocation. *Limnology and Oceanography* 28: 667-676.

Tessier AJ & Goulden CE (1987). Cladoceran Juvenile Growth. *Limnology and Oceanography* 32: 680-686.

Tessier AJ & Consolatti NL (1991). Resource quantity and offspring quality in *Daphnia*. *Ecology* 72: 468-478.

Tessier AJ, Leibold MA & Tsau J (2000). A fundamental trade-off in resource exploitation by *Daphnia* and consequences to plankton communities. *Ecology* 81:826-841.

Tessier AJ, Bizina EV & Geeday CK (2001). Grazer-resource interactions in the plankton: are all daphniids alike? *Limnology and Oceanography* 46: 1585-1595.

Tessier AJ & Woodruff P (2002). Cryptic trophic cascade along a gradient of lake size. *Ecology* 83: 1263-1270.

Tillmann U & Lampert W (1984). Competitive ability of differently sized *Daphnia* species: an experimental test. *Journal of Freshwater Ecology* 2: 311-323.

Tilman D (1980). Resources: a graphical-mechanistic approach to competition and predation. *American Naturalist* 116: 362-393.

Trubetskova I & Lampert W (1995). Egg size and egg mass of *Daphnia magna*: response to food availability. *Hydrobiologia* 307: 139-145.

Vanni MJ & Lampert W (1992). Food quality effects on life history traits and fitness in the generalist herbivore *Daphnia*. *Oecologia* 92: 48-57.

Capítulo III

Efeitos da disponibilidade de alimento na toxicidade do herbicida Propanil em *Daphnia* spp.

NOTA PRÉVIA: O capítulo III corresponde a um artigo científico que se encontra publicado numa revista internacional com arbitragem científica [*Ecotoxicology and Environmental Safety* 68: 386-396 (2007)]. Encontra-se, portanto, inteiramente escrito em língua inglesa, pelo que previamente se apresenta um resumo traduzido do trabalho constante do capítulo, acompanhado de uma lista de palavras-chave que a ele estão associadas.

Resumo | O aumento generalizado da aplicação de pesticidas na produção agrícola conduz frequentemente à contaminação de ecossistemas de água doce situados nas proximidades de campos agrícolas. Herbicidas, como o Propanil, podem induzir efeitos deletérios no *fitness* das populações zooplanctónicas (e.g. cladóceros) que, por sua vez, é já naturalmente influenciado pela disponibilidade de recursos alimentares e/ou pela eficiência na aquisição desses recursos pelos organismos. O objectivo central deste trabalho foi a avaliação das respostas individuais e populacionais de *Daphnia magna* e de três linhagens clonais distintas pertencentes ao complexo *Daphnia longispina*, a exposições agudas e crónicas do herbicida Propanil. Foi ainda abordado o potencial da disponibilidade de alimento na modelação das respostas de *Daphnia* em função da concentração de tóxico a que foram expostas (exposições crónicas). Os resultados obtidos demonstraram que o herbicida exibiu toxicidade aguda e crónica assinalável tanto para *D. magna* como para as populações de *Daphnia* cf *longispina*, sempre em gamas de concentração bastante baixas. Foram também observadas diferenças relevantes na resposta quer quando comparando os diferentes clones de *D.* cf *longispina* entre si, quer quando comparando estes últimos com *D. magna*. O nível alimentar (disponibilidade de recursos alimentares) demonstrou ser um factor condicionante do *fitness* exibido por *Daphnia* nos ensaios, muito embora não parecendo interferir especificamente com o modo de acção do tóxico.

Palavras-chave | *Daphnia magna*; *Daphnia* cf *longispina*; recursos alimentares; toxicidade de herbicidas; Propanil; viabilidade dos ovos.

Short- and long-term responses of *Daphnia* spp. to Propanil exposures in distinct food supply scenarios.

Joana Luísa Pereira, Cristiana Duarte Mendes & Fernando Gonçalves

CESAM& Departamento de Biologia da Universidade de Aveiro, 3810-193 Aveiro, Portugal.

Abstract | The widespread increase of pesticides application in crops frequently leads to the contamination of vicinal freshwater lentic ecosystems. Herbicides such as propanil may impair cladoceran fitness, which is per se strongly influenced by the food availability and/or its acquisition efficiency. This work intended to evaluate the responses of *Daphnia magna* and three clonal lineages belonging to the *Daphnia longispina* complex to acute and chronic exposures of the herbicide propanil, as well as to assess whether food availability features these responses. Results showed that the agrochemical was acutely and chronically toxic to both *D. magna* and the *D.* cf *longispina* clones at the same range of low concentrations, while relevant differences were depicted between the three distinct genotypes belonging to the D. longispina complex. Food-level conditioned the general fitness of the daphnids in the tests but evidences suggest that it does not interfere specifically with the toxicant mode of action.

Key-words| *Daphnia magna*; *Daphnia* cf *longispina*; food-level; herbicide toxicity; propanil; egg viability

INTRODUCTION

The worldwide use of pesticides in agriculture in the control of destructive insects, weeds and pathogens contributed to the raise of concerns on the contamination of surface- and ground-water bodies. Pesticides often reach lentic ecosystems by direct occasional spray drift, by drainage or even by leaching (Cerejeira et al. 2003). Due to secondary hazard properties, which are generally enhanced by some lack of target-specificity, these anthropogenic toxicants may interfere in one or several levels of the aquatic food-web. Even herbicides, which should be specifically designed to kill plant-weeds, are reported in literature as hazardous at low concentrations, both to aquatic vertebrates and invertebrates (e.g. Moore et al. 1998). In this way, the analysis of the responses to herbicide toxicity scenarios in the lower levels of the food web (i.e. phytoplankton and zooplankton) is considered to be informative of relative impacts in the aquatic ecosystem (Islam & Tanaka 2004). Assuming its key-position in the trophic interactions of the lentic ecosystems and considering its recognised high sensitivity to toxicants, zooplankton is frequently used in ecotoxicological

assessments (Hanazato 2001). Among zooplankters, the cladocerans, and specifically those belonging to the genus *Daphnia*, are an important food resource to planktivorous fish and therefore play an essential role in the energy transfer from primary producers to higher levels of the food web. Daphnids are easy to rear and handle in lab, and have a cyclically parthenogenic reproduction allowing highly controlled experimental designs. Moreover, individuals from the genus *Daphnia* have, among zooplankton, a relative higher sensitivity to toxicant stress, and thus their extensive use in ecotoxicology to evaluate general or more specific toxicity scenarios, such as those involving pesticide risk analyses (Hanazato 1998, 2001).

This work concerns the evaluation of the effects on *Daphnia* spp. of short- and long-term exposures to the herbicide Propanil. Belonging to the anilides chemical class (Orme and Kegley 2006), propanil (3,4-dichloropropioanilide) is a highly selective contact herbicide, which is commonly applied in the post-emergency of rice (*Oryza sativa*) to control grass- and broadleaf-weeds (Mitsou et al. 2006, Gómez de Barreda Ferraz et al. 2004). The World Health Organization recognized Propanil as slightly hazardous in terms of human risk (WHO 2004a), whereas, by admitting that Propanil environmental fate evolves its prompt degradation to highly hazardous metabolites such as 3,4-dichloroanilide (DCA), did not define any guideline reference concentration for the herbicide (WHO 2004b). Still, Orme and Kegley (2006), mainly based on USEPA reports, framed Propanil as slightly to highly toxic for zooplankton. In fact, residues of Propanil have been already detected in several surface water bodies (e.g. Albanis et al. 1998, Cerejeira et al. 2003) and have been found in paddy water samples within a considerable day-after-treatment (DAT) period (14 DAT *sensu* Perera et al. 1999), a problem that reasonably raises concerns on the potential of Propanil to harm aquatic ecosystems. Additionally, Propanil is highly water-soluble and is not likely to be strongly adsorbed to soil particles (low K_{oc}) (Albanis et al. 1998), thus enhancing the possibility for it to reach aquatic ecosystems near crops where applications occurred.

Phytoplankton densities in lentic ecosystems often fluctuate, and planktonic grazers such as *Daphnia* are known to deal with those fluctuations by changing their life-history performances to optimally allocate the available resources (Gliwicz & Guisande 1992, Boersma 1997), which is recognised as an adaptive response compromising the population dynamics (Pieters & Liess 2006). On the other hand, coping with physiological stress, such as that induced by sub-lethal toxicant exposures, is energetically costly to daphnids (Smolders et al. 2005), and often induces compensatory changes in their energetic metabolism (Smolders et al. 2005, DeCoen & Janssen

2003). Food availability, among other natural stressors, is indeed known to modulate the effects of pesticides in cladocerans (Hanazato 2001): the impairment of the feeding behaviour (filtering and/or ingestion ability) or the indirect toxicity that arises by incorporation of the toxic in the algae are examples of the mechanisms behind this pesticide-food interaction (Allen et al. 1995). Considering that food constraints have a critical role in the ability of daphnids to cope with chemical stress, it would be relevant to take them into account in ecotoxicological assays. Besides, several authors have additionally compromised the ecological relevance of responses assessed in bioassays and ecotoxicological assays, with the species and or the genotypes used (Antunes et al. 2003, 2004, Marques et al. 2004a,b). Differences in response to exposures either to toxicants or to natural stressors between genotypes belonging to the same species, due to their differential flexibility of life-history traits (e.g. phenotypic plasticity), were also recorded in several studies (Baird et al. 1989, Baird et al. 1990, Soares et al. 1992, Ebert 1993, Barata & Baird 1998).

With this study we intended to assess the extent of the acute and the chronic toxicity of the herbicide Propanil to a standard cladoceran (*D. magna*) and to three different genotypes belonging to the *D. longispina* complex, as well as to comparatively analyse the differences in sensitivity between species/*taxa* and between genotypes. The effects of the herbicide in *Daphnia* spp. life-history traits and population growth were evaluated under three food-levels, in order to check whether food availability can influence or modulate the chemical toxicity.

MATERIAL & METHODS

Test organisms

The monoclonal *Daphnia* spp. bulk cultures were continuously reared in the lab, under a 16:8 hr light:dark photoperiod, at a temperature of 20±2ºC, in synthetic ASTM hardwater medium (ASTM 1980) supplied with an organic additive extracted from the algae *Ascophyllum nodosum* (Baird et al. 1989). Cultures were renewed every other day and the organisms were fed with *Pseudokirchneriella subcapitata* (also cyclically cultured in lab according with Stein 1973) at a concentration of $3.0x10^5$ cells/mL and $1.5x10^5$ cells/mL for *Daphnia magna* (clone A, *sensu* Baird et al. 1989) and *Daphnia* c.f. *longispina* clones, respectively. The three *D.* c.f. *longispina* clonal lineages used in tests were established from field-collected samples: (1) clone M was collected in lake Mira (Mira, centre-northwest of Portugal) and has been maintained in the lab since 2001 (clone EM7

sensu Antunes et al. 2003); (2) clone V resulted from a sample picked in lake Vela (Quiaios, centre-northwest of Portugal) in 2004; (3) clone T was collected in the shallow reservoir Tapada Grande (Mértola, south-east of Portugal) in 2004. ITS-RFLP techniques (Billiones et al. 2004) confirmed that these clones represent three distinct *taxa* within the *D. longispina* complex (Petrusek et al. 2005).

Test procedures

A Propanil stock solution was obtained by direct dilution of its commercial formulation Stam Novel Flow 480® - 480 g/L Propanil concentrated (Sow, Portugal) - in distilled water and stored at 4ºC in dark. All the test solutions were freshly prepared by diluting the appropriate volumes of the stock solutions in ASTM hardwater. Acute or the life-history assays were carried independently for each *Daphnia* population in general accordance with respective standardized protocols (OECD 1998, 2000) and under the incubation conditions previously described for cultures. Seeking the reduction of maternal effects, only neonates born between the third and the fifth broods (< 24-hr old) were used in tests (Barata & Baird 1998).

A static approach along 48-hrs, consisting in the exposure of the *Daphnia* populations to six nominal Propanil concentrations plus one ASTM negative control without food or organic additive supply, was applied to each of the acute assays. Twenty newborns per treatment (four replicates with five animals each) were used. Dissolved oxygen and pH were monitored at the beginning and the end of the tests for validation purposes. Tests were screened for immobilised individuals at 24- and 48-hr exposure period, and records were used for further EC_{50} calculations (see *Statistics*).

Several preliminary chronic tests were carried until the establishment of a suitable final range of test-concentrations for the accomplishment of valid fecundity data in reproduction assays. In the chronic experiments, the daphnids were individually exposed along 21 days to five nominal concentrations of Propanil plus one ASTM negative control (10 replicates per treatment). Renewal occurred every other day to freshly prepared medium or test solution. The life-history experiments followed a bifactorial design, where the four populations (*D. magna* and *D. longispina* M, V and T) were exposed to Propanil at three different daily food rations (i.e. *P. subcapitata* at low, middle and high level): $0.750x10^5$, $1.500x10^5$ and $3.000x10^5$ cells/mL for *D. magna*; $0.375x10^5$, $0.750x10^5$, $1.500x10^5$ cells/mL for the three *D.* c.f. *longispina* populations. Dissolved oxygen and pH were monitored along the test for validation purposes.

Tests were checked daily for eventual mortality and for viable or non-viable progeny. When present, offspring were counted and immediately removed, allowing the direct calculation of the viable fecundity (total offspring number), the total fecundity (offspring number + non-viable eggs and/or embryos) and the age of females at first reproduction. The body size of the females was estimated at the beginning and at the end of the test, by extrapolation from the exopodite length of the moulted exuvia (Pereira et al. 2004).The somatic growth (*g sensu* Burns 2000) of the females was then calculated:

$$g = [\ln(l_f) - \ln(l_0)] / \Delta t \quad (day^{-1}),$$

where l_f is the final body length (mm), l_0 is the initial body length (mm) and Δt is the time range (days). Fecundity data and the eventual mortality occurrence were integrated for the estimation of the per capita rate of population increase (r) through the Euler- Lotka equation:

$$1 = \sum_{i=1}^{n} e^{rx} l_x m_x$$

where r stands for the *per capita* rate of population increase (day^{-1}), x for the age class (days), l_x for the probability of surviving to age x (0…n), and m_x for the fecundity at age x. Uncertainties were estimated according to the Jackknife technique (Meyer et al. 1986).

Statistics

Probit analysis (Finney 1971) allowed the calculation of the 48-hr EC_{50} immobilisation values with the respective 95% confidence limits, for each population. For each *Daphnia* population, a two-way analysis of variance (two-way ANOVA) was used to assess the significance of the effects of food-level and propanil concentration, as well as their interaction, on each of the life-history endpoints and on the population growth estimate. As highly significant interactions were nearly always detected, a one-way ANOVA, followed by the applicable *post-hoc* multi-comparisons test, was carried out to achieve differences in propanil treatments relatively to the control within each food-level (Zar 1996; Quinn and Keough 2002). A significance level (α) of 0.05 was used in both ANOVA approaches. The statistical significance of the production of non-viable progeny was checked by testing the viable fecundity against the total fecundity within each propanil concentration. A paired Wilcoxon test with an adjusted significance level (Dunn-Sidak procedure: $P \leq 0.009$) was used for this purpose (Quinn & Keough 2002).

RESULTS

Either the acute or the chronic assays fulfilled the validity criteria recommended in the standardised protocols (OECD 1998, 2000). Dissolved oxygen and pH did not fluctuate during the assays or with the increase of toxicant concentrations (min-max: 7.2-8.4 mg/L O2 and 7.60-8.20 pH).

Regarding the acute toxicity, the 48-hr EC_{50} determinations positioned *Daphnia magna* as the most sensitive species (EC_{50}= 3.55 mg/L). The *Daphnia* c.f. *longispina* populations presented slightly higher tolerances, but similar among them (6 mg/L< EC_{50}< 8 mg/L, with overlapping 95%-confidence limits) (FIGURE III.1). Despite this narrow interval, it was on *D. longispina* V where Propanil was less toxic (EC_{50}= 7.51 mg/L) and on *D. longispina* M where the chemical was most toxic. The chronic assays were firstly performed comprising ranges of concentrations immediately below each EC_{50}. Although no mortality occurred during the 21 days of these preliminary tests, no viable offspring were released, i.e., despite we confirmed egg production, the offspring never completed their development even in the lower Propanil treatments. Several sequential reductions in concentration ranges had to be carried out until the females started releasing viable progeny, which indicates *per se* that Propanil had a much higher chronic toxicity to *Daphnia* spp. than what would be expectable from the determined 48-hrs EC_{50}s.

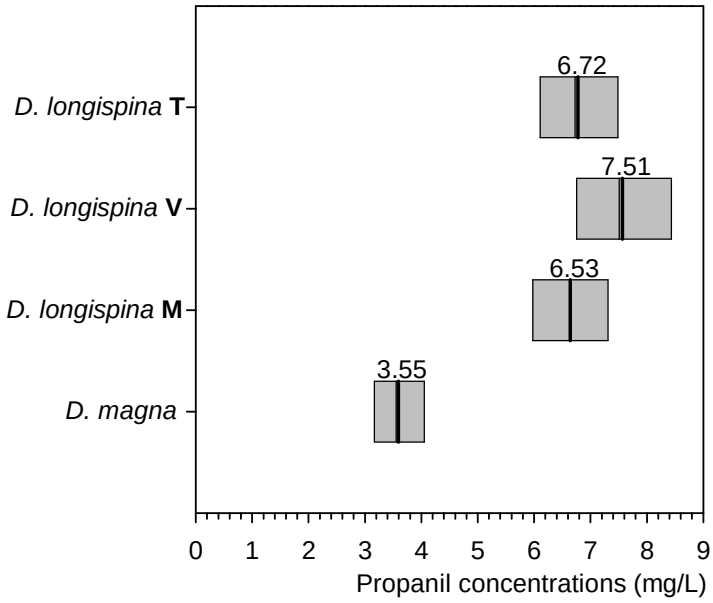


FIGURE III.1. | Immobilisation 48-hr EC_{50} values (the actual value is specified in the top of each box in mg/L) and respective 95% confidence limits, for *D. magna* and for the three populations of *D.* cf *longispina*.

Mortality was low in all the chronic assays, never surpassing 20% in any treatment, and the occasionally observed records were not compromised with the toxicant increase. The impairment caused by the toxic on the reproductive parameters as well as on the population and somatic growth rates was generally maintained when comparing the responses between food levels: i) there was in fact a trend for a better relative performance on the life-history endpoints and on the population growth estimates when food supply rises (FIGURE III.2 and III.3); ii) although, the testing for differences between the toxic treatments (TABLE III.I) and also the statistically significant differences relatively to control (FIGURE III.2 and III.3) suggest a broadly similar pattern for the Propanil mode of action when comparing among food treatments. Conversely, the two-way ANOVA denoted only very few exceptions to the detection of statistically highly significant interactions between the food and the Propanil treatments (TABLE III.II); and, when looking at the variance partitioning, a conspicuously higher contribution of the food factor relatively to that of the Propanil factor is noticeable (TABLE III.II).

TABLE III.I: One-way ANOVA summary regarding the life-history responses of the *Daphnia* populations to the propanil exposure within food-level. Six propanil treatments including the negative control were tested. df – among group, residual degrees of freedom.

	Endpoint	Food-level	df	$MS_{residual}$	*F* ratio	*P* value
D. magna	Viable fecundity	low	5, 53	11.90	11.570	<0.001
		middle	5, 54	337.0	18.574	<0.001
		high	5.53	206.8	1.921	0.106
	Growth Rate	low	5, 53	$1.25\ e^{-7}$	93.55	<0.001
		middle	5, 54	$5.74\ e^{-6}$	14.71	<0.001
		high	5, 53	$3.80\ e^{-6}$	4.014	0.004
	Age at first reproduction	low	5, 53	0.337	0.669	0.648
		middle	5, 54	0.856	19.11	<0.001
		high	5, 53	0.774	0.962	0.449
	r	low	5, 54	$2.65\ e^{-4}$	4.304	0.002
		middle	5, 54	$9.09\ e^{-4}$	36.89	<0.001
		high	5, 54	$7.86\ e^{-4}$	4.116	0.003
***D. longispina* M**	Viable fecundity	low	5, 48	6.528	5.944	<0.001
		middle	5, 53	11.55	48.30	<0.001
		high	5, 47	19.71	12.58	<0.001
	Growth Rate	low	5, 49	$1.76\ e^{-6}$	3.162	0.015
		middle	5, 53	$5.24\ e^{-19}$	0.000	1.000
		high	5, 47	$4.01\ e^{-6}$	7.941	<0.001
	Age at first reproduction	low	5, 48	2.652	1.209	0.319
		middle	5, 53	1.160	1.748	0.140
		high	5, 47	1.211	2.570	0.039
	r	low	5, 54	$1.53\ e^{-3}$	0.562	0.729
		middle	5, 54	$6.49\ e^{-4}$	14.76	<0.001
		high	5, 54	$7.81\ e^{-4}$	11.38	<0.001
***D. longispina* V**	Viable fecundity	low	5, 45	21.83	6.004	<0.001
		middle	5, 51	17.42	33.32	<0.001
		high	5, 50	51.75	30.52	<0.001
	Growth Rate	low	5, 45	$7.15\ e^{-7}$	25.94	<0.001
		middle	5, 51	$1.63\ e^{-18}$	0.000	1.000
		high	5, 50	$2.51\ e^{-6}$	8.054	<0.001
	Age at first reproduction	low	5, 42	2.287	3.200	0.015
		middle	5, 50	5.682	2.409	0.049
		high	5, 49	2.210	1.941	0.105
	r	low	5, 54	$1.94\ e^{-3}$	7.776	<0.001
		middle	5, 54	$2.06\ e^{-3}$	4.979	<0.001
		high	5, 54	$2.66\ e^{-3}$	10.41	<0.001

***D. longispina* T**	Viable fecundity	low	5, 47	8.205	6.366	<0.001
		middle	5, 49	35.97	6.541	<0.001
		high	5, 47	61.04	16.64	<0.001
	Growth Rate	low	5, 47	4.64 e^{-6}	1.860	0.120
		middle	5, 49	6.04 e^{-6}	0.432	0.824
		high	5, 47	6.47 e^{-6}	1.330	0.268
	Age at first reproduction	low	5, 46	4.797	1.295	0.282
		middle	5, 49	0.100	1.618	0.173
		high	5, 47	0.220	0.775	0.573
	r	low	5, 54	2.96 e^{-3}	1.435	0.227
		middle	5, 54	1.06 e^{-3}	5.778	<0.001
		high	5, 54	9.89 e^{-4}	3.843	0.005

TABLE III.II: Two-way ANOVA summary relative to the life-history responses of the *Daphnia* populations exposed to propanil at different food-levels. Six propanil treatments including the negative control and three food-levels were considered in the analysis. df - among group, residual degrees of freedom.

	Endpoint	**Source of variation**	**df**	**MS**	***F* ratio**	***P* value**
D. magna	Viable fecundity	Food	2, 160	3.8310	278.56	<0.001
		Propanil	5, 160	0.4770	34.693	<0.001
		Food x Propanil	10, 160	0.1840	13.378	<0.001
	Growth Rate	Food	2, 160	1.38 e^{-4}	42.513	<0.001
		Propanil	5, 160	8.81 e^{-5}	27.229	<0.001
		Food x Propanil	10, 160	1.13 e^{-5}	3.503	<0.001
	Age at first reproduction	Food	2, 160	33.383	50.832	<0.001
		Propanil	5, 160	7.0180	10.686	<0.001
		Food x Propanil	10, 160	4.9430	7.526	<0.001
	r	Food	2, 162	0.0867	132.79	<0.001
		Propanil	5, 162	0.0220	33.752	<0.001
		Food x Propanil	10, 162	0.0079	12.133	<0.001
***D. longispina* M**	Viable fecundity	Food	2, 148	5559.4	444.29	<0.001
		Propanil	5, 148	600.57	47.996	<0.001
		Food x Propanil	10, 148	101.49	8.1110	<0.001
	Growth Rate	Food	2, 149	3.29 e^{-4}	178.23	<0.001
		Propanil	5, 149	1.77 e^{-5}	9.5960	<0.001
		Food x Propanil	10, 149	1.07 e^{-5}	5.7730	<0.001
	Age at first reproduction	Food	2, 148	105.37	63.473	<0.001
		Propanil	5, 148	4.3150	2.5990	0.028
		Food x Propanil	10, 148	1.2370	1.2370	0.272
	r	Food	2, 162	0.2220	225.44	<0.001
		Propanil	5, 162	0.0109	11.062	<0.001
		Food x Propanil	10, 162	0.0042	4.2670	<0.001
***D. longispina* V**	Viable fecundity	Food	2, 146	4519.4	281.16	<0.001
		Propanil	5, 146	1761.3	109.57	<0.001
		Food x Propanil	10, 146	250.21	15.565	<0.001
	Growth Rate	Food	2, 146	4.21 e^{-4}	390.77	<0.001
		Propanil	5, 146	2.48 e^{-5}	22.995	<0.001
		Food x Propanil	10, 146	7.27 e^{-6}	6.7470	<0.001
	Age at first reproduction	Food	2, 141	23.387	6.7510	0.002
		Propanil	5, 141	18.169	5.2450	<0.001
		Food x Propanil	10, 141	2.8250	0.8150	0.614
	r	Food	2,162	0.1590	71.730	<0.001
		Propanil	5, 162	0.0486	21.870	<0.001
		Food x Propanil	10, 162	0.0023	1.014	0.434
***D. longispina* T**	Viable fecundity	Food	2, 143	18.588	529.78	<0.001
		Propanil	5, 143	904.89	25.791	<0.001
		Food x Propanil	10, 143	185.96	5.3000	<0.001
	Growth Rate	Food	2, 143	2.25 e^{-5}	3.9380	0.022
		Propanil	5, 143	1.19 e^{-5}	2.0830	0.071
		Food x Propanil	10, 143	4.01 e^{-6}	0.702	0.722
	Age at first reproduction	Food	2, 142	153.17	92.203	<0.001
		Propanil	5, 142	2.431	1.463	0.206
		Food x Propanil	10, 142	2.167	1.304	0.234
	r	Food	2, 162	0.4900	293.70	<0.001
		Propanil	5, 162	0.0096	5.7340	<0.001
		Food x Propanil	10, 162	0.0023	1.3830	0.192

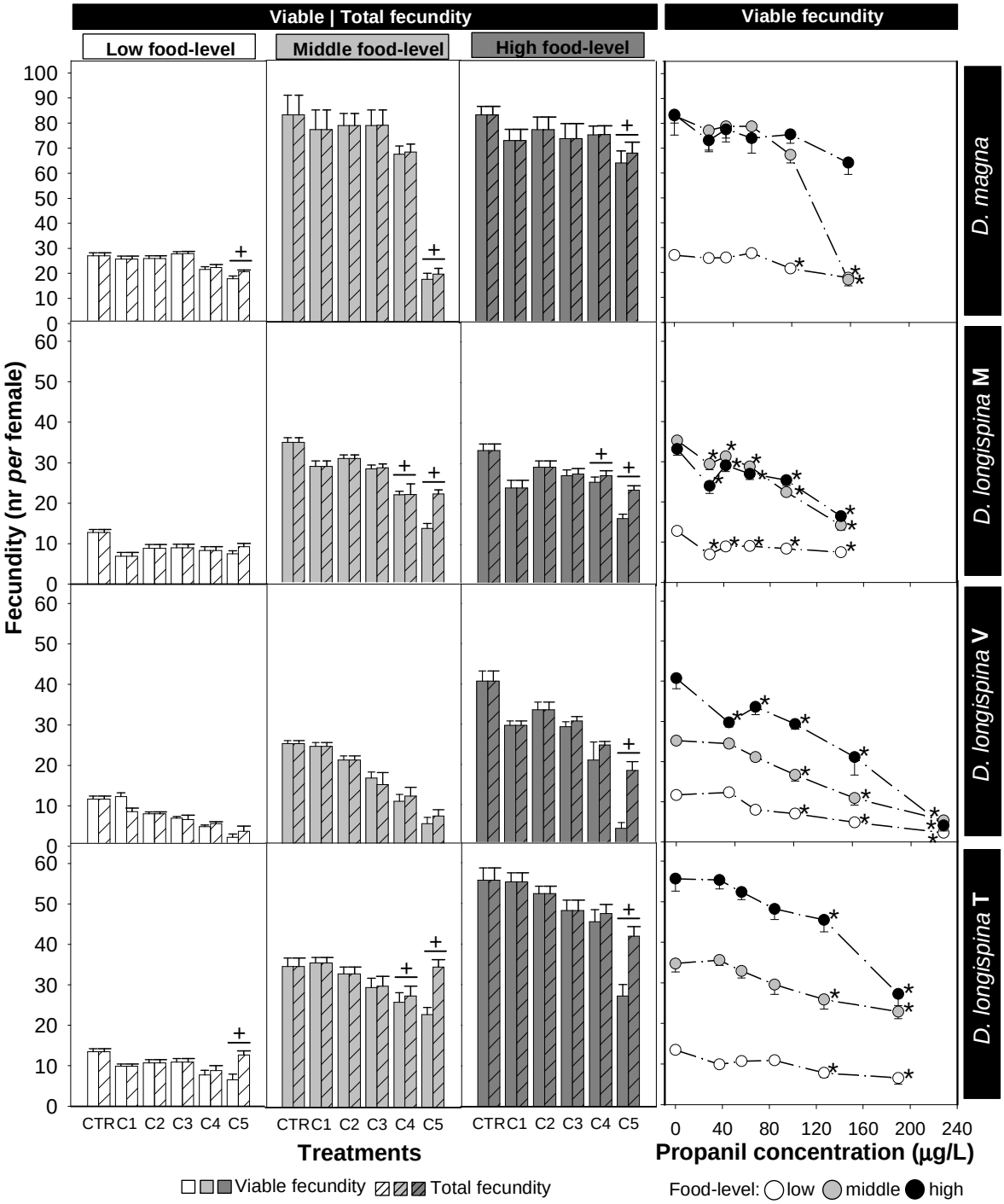


FIGURE III.2 | Fecundity records for the four *taxa* exposed to Propanil at the different food-levels. Bar charts represent viable and total fecundity at each food-level x *taxon*; the symbols CTR and C1-C5 (which are discriminated on the Propanil nominal concentration scale) represent the negative control and the five Propanil tested concentrations, respectively. The symbol '+' represents statistically significant differences between viable- and total offspring number within each Propanil concentration (paired Wilcoxon test, P≤0.009). Line plots show the viable fecundity profiles along the Propanil concentration ranges for each food-level, and associated significances relative to the control (P≤0.05). Error bars represent always the standard error.

Excluding *D. magna* in the high food-level (H), the viable fecundity (i.e. the total output of offspring per female) was always significantly affected by Propanil (TABLE III.1). *D. magna* was more tolerant than the *D. longispina* populations within all the food levels; within the *D. longispina* populations, the clone T presented the higher tolerance to the toxic, given that statistically significant impairment on the reproductive output was only depicted above a 120 µg/L Propanil exposure in the three food-levels; very low concentrations of Propanil (*ca* 30 µg/L) significantly decreased the fecundity of *D. longispina* M, despite the food-level (FIGURE III.2 – viable fecundity). When comparing the viable fecundity decrease between food treatments, a stronger effect of the toxic was found in the higher food-levels: *D. longispina* V and T provide the better picture of this trend. The total fecundity (i.e. the cumulative output of offspring and non-viable eggs and embryos) was also reduced by increasing concentrations of Propanil (FIGURE III.2 – Viable | Total fecundity). Differences between the viable and the total fecundity in the higher Propanil treatments were invariably detected (paired Wilcoxon test; $p \leq 0.009$), with the few exceptions of the low (L) and the middle (M) food-levels of *D. longispina* V. Even accounting for these latter exceptions, the adverse effect of the toxic observed on the viable fecundity tends to became smoothed when compared to that observed on the total fecundity. In fact, this is especially emphasized for L and M food-levels in *D. longispina* T: at the higher Propanil concentrations there was a statistically significant decrease (Dunnet test; $p \leq 0.05$) in viable fecundity, whereas the total fecundity achieved records within the range of those found for the controls (FIGURE III.2 – Viable | Total fecundity).

In general, and within each food level, the somatic growth rates and intrinsic rates of population increase (*r*) were significantly impaired by Propanil treatments (FIGURE III.3; TABLE III.I). Despite the visibly decreasing trend with increasing Propanil concentration, the somatic growth rate seems to be the less responsive parameter given that statistics did not reveal a consistent picture in the responses within species or within food-levels. A consistent, statistically significant decrease in *r* was observed for all the species and within all food-levels, with a single exception for *D. longispina* M in L. Concomitantly, one can denote a general tendency for a slight delay in reproduction with the exposure to higher Propanil concentrations, although not always statistically significant (FIGURE III.3; TABLE III.I). Within all the *Daphnia* populations, the lower food-level shows the lower *r* estimates and the greater delay in age at first reproduction; when the food supply increases, *r* tended to consecutively achieve higher values (*r* in M < *r* in H), while earlier reproduction occurred in M and H broadly in the same range. Moreover, a stronger impact of the increasing toxic levels in both the parameters' profiles is noticeable at the higher food-levels.

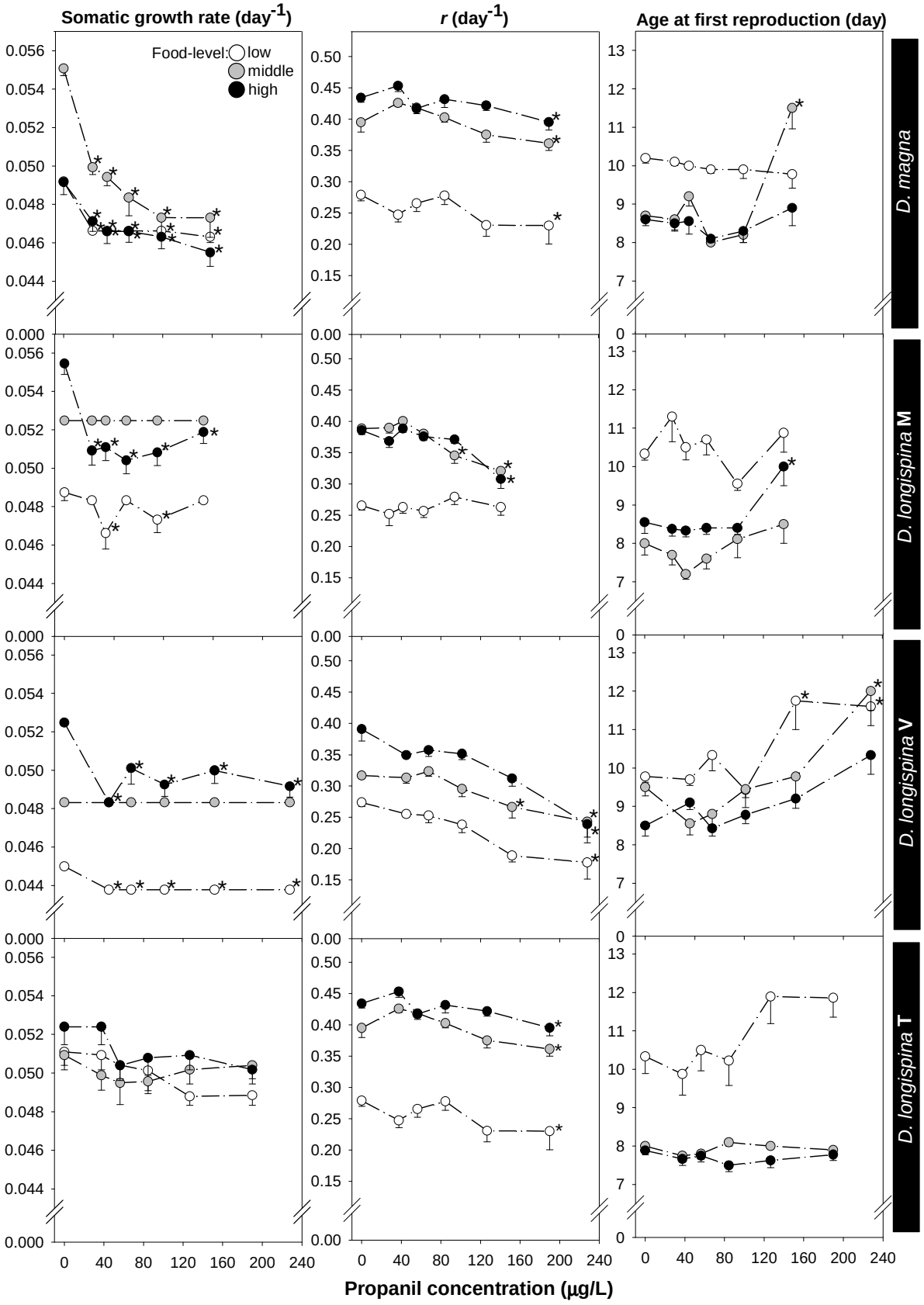


FIGURE III.3 | Life-history endpoints of *Daphnia* spp. exposed to propanil at different food-levels. Error bars represent the standard error and '*' stands for statistically significant differences in each trait, relative to the control (P≤0.05).

Although the chronic exposures pictured Propanil as a very toxic chemical to the tested organisms, a clear disposition of species as to their sensitivity could not be established. Results allow to position *D. longispina* M as the most sensitive species except for the somatic growth rate, whereas *D. magna* presented significant reductions at lower Propanil concentrations. *D. longispina* V and *D. longispina* T were the most tolerant species when regarding the somatic growth rate, *r*, and age at first reproduction.

DISCUSSION

Despite its widespread use in rice crops worldwide, little can be found in the literature on Propanil toxicity to non-target aquatic organisms, particularly to daphnids. Regarding the acute toxicity of the chemical, Villarroel et al. (2003) found a Propanil 48-hr LC_{50} of 5.01 mg/L for *Daphnia magna* while Pereira et al. (2000) obtained a lower value for the same endpoint (between 2.67 and 3.67 mg/L) by using a Daphtoxkit® in natural waters fortified with the herbicide. The smaller cladoceran *Ceriodaphnia dubia* was less tolerant to the chemical registering a 48h-LC_{50} of 1.65 mg/L (Moore et al. 1998). Although respecting the same order of magnitude, our immobilisation EC_{50} values were relatively distinct from these literature data. However, the relative higher tolerance of our organisms to the toxic can not be strictly assumed since: (i) The culture methods, the used genotypes and the technical specificities in the assessment methods chosen by those authors do not exactly match with ours, therefore constraining these comparisons; (ii) Previous work in our lab (Mendes et al. 2007) showed that Stam Novel Flo 480® was less acutely toxic to *D. magna* than its active ingredient Propanil, and actually found a 48-hr EC_{50} for technical-grade Propanil quite similar to those obtained by the mentioned authors. Moreover, in our acute assays, *D. magna* showed an unexpected lower tolerance to Propanil than the *D.* cf *longispina* clones. The smaller size of the latter species, and the consequent greater surface-to-volume ratio (Lilius et al. 1995), would theoretically expose more the organism to the waterborne chemical. This pattern has been verified by several authors when comparing acute toxicities of different chemicals between *D. magna* and *D. longispina* (Antunes et al. 2004, Marques et al. 2004a,b). The acute assays' outcome denoting a higher sensitivity of the larger species was not confirmed in the chronic exposures.

The egg/embryo mortality observed at very low toxicant concentrations, and the apparent buffering-effect that exists when regarding the total *vs* the viable fecundity (see FIGURE III.2), seem to indicate a direct contamination pathway via body surface. Villarroel et al. (2003) also reduced largely

their toxic concentrations (relatively to those they used to determine the EC_{50} value) when establishing the sub-lethal range for the chronic assays with *D. magna*, but there are no references to the production of non-viable progeny. Effects on egg development were already reported for both *D. magna* and *D. longispina* exposures to the Propanil metabolite 3,4-Dichloroaniline (DCA) (Baird et al. 1991, Barata & Baird 2000, Beyerle-Pfnür 1991, Trubetskova & Lampert 2002), as a consequence of a direct (*via* surface-mediated exchanges) and specific egg poisoning in the brood pouch, remaining the eggs enclosed in the ovaries intact (Baird et al. 1991). Propanil has a reported hydrolysis half-life of 5000 days (Orme & Kegley, 2006) and there are evidences that its complete breakdown occurs in *ca* 12 months in natural ground-water samples kept in dark at environmental temperature conditions (Cavalier et al. 1991). Regarding that these time ranges for degradation are likely to be highly accelerated in surface waters (Cavalier et al. 1991), effects on egg development could be admissible to be due to the presence of DCA, resulting from Propanil direct breakdown. However, our stock solutions (obtained by dilution in distilled water) were kept in dark refrigerated conditions and the test solutions were renewed every other day, which slows the degradation pathway. Thus, it is likely that the teratogenic effects were caused by Propanil itself. In spite of the lack of data on the subject for aquatic invertebrates, hatchability impairment was already observed on *Pimephales promelas* after a low-level Propanil exposure (Call et al. 1983 *in* Desfuly et al. 2003).

The lower relative tolerance of eggs when compared to *Daphnia* adults in toxic exposures has already been documented. Baird et al. (1991) has found DCA and sodium bromide as more toxic to eggs than to *D. magna* adults and Sobral et al. (2001) reported the same pattern concerning exposures to an organochlorine compound, a surfactant, two metals and a complex industrial effluent. Abe et al. (2001) has found a higher sensitivity of embryos than adults to chlorinated anilines. Taking into account what was suggested by Baird et al. (1991) on DCA lethal effects in eggs, it is reasonable to hypothesize that Propanil may not enter in the carapace of the adult female, but easily affect the eggs in the brood pouch, which is in direct communication with the external medium in order to assure gas exchanges. Aromatic anilides such as Propanil are known to induce an increase in methemoglobin content in several aquatic species (Crossland 1990). Moreover, propanil seems to be able to bind hemoglobin and induces the raise of methemoglobin levels in rat blood cells (McMillan et al. 1990). In parallel to what was discussed by Guilhermino et al. (1999) addressing DCA-induced egg abortion in *D. magna*, one can hypothesize that as oxygen levels are lower in the brood pouch than in the external medium surrounding the adult female, an induction of

the methemoglobin production via direct exposure to Propanil can be critical for the eggs/embryos development. Nevertheless, further research is needed in order to clarify these mechanisms.

The toxicant impairment on the life-history and population growth occurred in all *Daphnia* populations with a relative independence of the food-level, despite the frequent detection of interactions between factors (two-way ANOVA): regardless the resource constraints, daphnids were not able to keep fitness levels (similar to those of the control treatment). However, the way daphnids coped with Propanil differed between food-levels *ie* as food increases there is a general trend to a most serious effect of the toxic in the daphnids' fitness and hence the detection of highly significant interactions between those factors. *Daphnia* life-history traits flexibility allows individuals to take the better advantage of any situation in terms of food quantity. When facing food depressions, daphnids broadly retard their growth and thus smaller females reproduce later, releasing smaller broods of larger, more hunger-resistant, neonates (Gliwicz & Guisande 1992, Trubetskova & Lampert 1995, Boersma 1997, Smolders et al. 2005). In high-food environments, daphnids allocate more energy resources to reproduction, often at the expenses of self-maintenance and thus stress resistance, while in low-food conditions their investment in self-maintenance is preferred to that in reproduction, which generally provides an increase in stress resistance (Smolders et al. 2005, Polishchuk & Vijverberg 2005). Our results show that, as food availability raises, the reproductive traits achieve better records either in control or in the toxicant treatments, which indicates a growing effort to allocate the available resources to reproduction. However, both the viable fecundity profiles and age at first reproduction profiles (see FIGURE 2 and 3) of our *Daphnia* populations generally reflect a relative lower impact of the toxic in the low food-level, which suggests that in poor food conditions the organisms cope more efficiently with the toxic stress than in better food conditions.

In face of a certain environmental stimulus, the iteroparous *Daphnia* changes individually its fitness in order to achieve the best contribution for the population "health" (Polishchuk & Vijverberg 2005, Pieters & Liess 2006). In this context, the population growth rate (*r*), as an integrative parameter, combines records regarding both lethal and sub-lethal effects of toxicity, therefore providing an ecologically more relevant estimate (Forbes & Calow 1999). Propanil exposures in between 130 and 140 µg/ L promoted a significant decrease in the *r* of the tested species suggesting that the chemical is likely to have the ability to structurally affect the population at low concentrations. Data indicate that, as mortality in tests was negligible, the general delay in the first reproduction joined with the clear reduction in the reproductive outputs were the major contributions for changes

on *r*. Although not directly, the egg/embryo mortality seems to be of importance to *r* estimates since its production was closely related with a decrease in viable offspring, which is by itself a meaningful endpoint in the calculation of that population parameter. Albeit seasonal variations, Propanil has been detected in Portuguese surface waters bodies (Cerejeira et al. 2003): the exact recorded concentrations are not available, which limits further inferences on the real potential of Propanil residues to harm *Daphnia* populations in lentic ecosystems based in our results.

The outcome of the chronic exposures did not allow a clear demarcation between species (*D. magna vs Daphnia* cf *longispina*) regarding their tolerance to Propanil. However, within the *Daphnia* cf *longispina* genotypes, there was a slight trend of the clone M to present higher sensitivities than the other clones when regarding either the life-history endpoints or *r*. Differences between species in tolerance can be of a critical influence in driving structural and functional adjustments as response to stress, in natural populations and communities (Hanazato 1998, 2001).

CONCLUSION

The herbicide Propanil was toxic to the non-target zooplankters *Daphnia* spp. at low (potentially more realistic) concentrations, particularly in long-term exposures. Corroborating what was suggested in the review by Hanazato (2001), in the context of the natural stressors' ability to influence the effects of pesticides in cladocerans, food availability modulated the Propanil effects in our *Daphnia* populations. This suggests that variations in food availability should be considered as a complementary factor in regular toxicity tests, especially when they are used as a tool in risk assessment of freshwater ecosystems. Moreover, data also confirmed that distinct *taxa* within the genus *Daphnia* respond differently either to the toxic or the food stress. This also compromises the ecological relevance of standard species' responses (recommended in ecotoxicological guidelines) to a toxicant stress.

REFERENCES

Abe T, Saito H, Niikura Y, Shigeoka T & Nakano Y (2001). Embryonic development assay with *Daphnia magna*: application to toxicity of aniline derivates. *Chemosphere* 45: 487-495.

Allen Y, Callow P & Baird DJ (1995). A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. *Environmental Toxicology and Chemistry* 14: 1625-1630.

Albanis TA, Hela DG, Sakellarides TM & Konstantinou IK (1998). Monitoring of pesticide residues and their metabolites in surface and underground waters of Imathia (N. Greece) by means of solid-phase extraction disks and gas chromatography. *Journal of Chromatography A* 823: 59-71.

Antunes SC, Castro BB & Gonçalves F (2003). Chronic responses of different clones of *Daphnia longispina* (field an *ephippia*) to different food-levels. *Acta Oecologica* 24: S325-S332.

Antunes SC, Castro BB & Gonçalves F (2004). Effect of food level on the acute and chronic responses of daphnids to lindane. *Environmental Pollution* 127: 367-375.

ASTM (1980). Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Report E 729-80. American Society for Testing and Materials, Philadelphia.

Baird DJ, Soares AMVM, Girling A, Barber I, Bradley MC & Calow P (1989). The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. *In:* H. Lokke, H. Tyle & F. Bro-Rasmussen (Eds.), Proceedings of the First European Conference on Ecotoxicology. Lyngby, pp. 144-148.

Baird DJ, Barber I & Callow P (1990). Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. I. Chronic life-history effects. *Functional Ecology* 4: 399-407.

Baird DJ, Barber I, Soares AMVM & Callow P (1991). An early life-stage test with *Daphnia magna* Straus: an alternative to the 21-day chronic test?. *Ecotoxicology and Environmental Safety* 22: 1-7.

Barata C & Baird DJ (1998). Phenotypic plasticity and constancy of life-history traits in laboratory clones of *Daphnia magna* Straus: effects of neonatal length. *Functional Ecology* 12: 442-452.

Barata C & Baird DJ (2000). Determining the ecological mode of action of chemicals from measurements made on individuals: results from instar-based tests with *Daphnia magna* Straus. *Aquatic Toxicology* 48: 95-209.

Beyerle-Pfnür R, Burkhardt G, Peither A & Lay JP (1991). Chronic ecotoxicity of 3,4 dichloroaniline to freshwater ecosystems. *Toxicological and Environmental Chemistry* 31-32: 367-373.

Billiones R, Brehm M, Klee J & Schwenk K (2004). Genetic identification of *Hyalodaphnia* species. *Hydrobiologia* 526: 43-53.

Boersma M (1997). Offspring size and parental fitness in *Daphnia magna*. *Evolutionary Ecology* 11: 439-450.

Burns CW (2000). Crowding-induced changes in growth, reproduction and morphology of *Daphnia*. *Freshwater Biology* 43: 19-29.

Call DJ, Brooke LT, Raymond JK, Knuth ML, Anderson C & Moriarity C (1983). Toxicity, bioconcentration and metabolism of the herbicide Propanil (3',4'-dichloropropionanilide) in freshwater fish. *Archives of Environmental Contamination and Toxicology* 12: 175-182.

Cavalier TC, Lavy TL & Mattice JD (1991). Persistence of selected pesticides in ground-water samples. *Ground Water* 29: 225-231.

Cerejeira MJ, Viana P, Batista S, Pereira T, Silva E, Valério MJ, Silva A, Ferreira M & Silva-Fernandes AM (2003). Pesticides in Portuguese surface and ground waters. *Water Research* 37: 1055-1063.

Crossland NO (1990). A review of the fate and toxicity of 3,4-dichloroaniline in aquatic environments. *Chemosphere* 21: 1489-1497.

De Coen WM & Janssen CR (2003). The missing biomarker link: relationship between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental Toxicology and Chemistry* 22: 1632-1641.

Desfuly BS, Giary L, Simoni E, Palazzi D & Manera M (2003). Alteration on rodlet cells in shub caused by the herbicide Stam M4® (Propanil). *Journal of Fish Biology* 63: 232-239.

Ebert D, Yampolski L & Van Noordwijk AJ (1993). Genetics of life-history in *Daphnia magna*. II. Phenotypic plasticity. *Heredity* 70: 344-352.

Finney DJ (1971). Probit analysis. Cambridge University Press, Cambridge. 350p.

Forbes VE & Calow P (1999). Is the per capita rate of increase a good measure of population-level effects in ecotoxicology?. *Environmental Toxicology and Chemistry* 18: 1544-1556.

Gliwicz ZM & Guisande C (1992). Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia* 91: 463-467.

Gómez de Barreda Ferraz D, Sabater C & Carrasco JM (2004). Effects of propanil, tebufenozide and mefenacet on growth of four freshwater species of phytoplankton: a microplate bioassay. *Chemosphere* 56: 315-320.

Guilhermino L, Sobral O, Chastinet C, Ribeiro R, Gonçalves F, Silva MC & Soares AMVM (1999). A *Daphnia magna* first-brood chronic test: an alternative to the conventional 21-day chronic bioassay?. *Ecotoxicology and Environmental Safety* 42: 67-74.

Hanazato T (1998). Response of a zooplankton community to insecticide application in experimental ponds: a review and the implications of the effects of chemicals on the structure and functioning of freshwater communities. *Environmental Pollution* 101: 361-373.

Hanazato T (2001). Pesticide effects on freshwater zooplankton: an ecological perspective. *Environmental Pollution* 112: 1-10.

Islam M & Tanaka M (2004). Impacts of pollution on coastal and marine ecosystem including coastal and marine fisheries and approach for management: a review and synthesis. *Marine Pollution Bulletin* 48: 624-649.

Lilius H, Hastbacka T & Isomaa B (1995). A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. *Environmental Toxicology Chemistry* 14: 2085-2088.

Marques CR, Abrantes N & Gonçalves F (2004a). Life-history traits of standard and autochtonous cladocerans: I. Acute and Chronic effects of acetylsalicylic acid. *Environmental Toxicology* 19: 518-526.

Marques CR, Abrantes N & Gonçalves F (2004b). Life-history traits of standard and autochtonous cladocerans: II. Acute and chronic effects of acetylsalicylic acid metabolites. *Environmental Toxicology* 19: 527-540.

McMillan DC, McRae TA & Hinson JA (1990). Propanil-induced methemoglobinemia and haemoglobin binding in the rat. *Toxicology and Applied Pharmacology* 105: 503-507.

Mendes CD, Pereira JL & Gonçalves F (2007). Acute and chronic effects of Stam Novel Flo 480® (commercial solution) and its active ingredient Propanil in *Daphnia magna*. *Fresenius Environmental Bulletin* 16: 537-542.

Meyer JS, Ingersoll CG, McDonald LL & Boyce MS (1986). Estimating uncertainty in population growth rates: Jackknife *vs* Bootstrap techniques. *Ecology* 67: 1156-1166.

Mitsou K, Koulianou A, Lambropoulou D, Pappas P, Albanis T & Lekka M (2006). Growth rate effects, responses of antioxidant enzymes and metabolic fate of the herbicide Propanil in the aquatic plant *Lemna minor*. *Chemosphere* 62: 275-284.

Moore MT, Pierce JR, Milam CD, Farris JL & Winchester EL (1998). Responses of non-target aquatic organisms to aqueous propanil exposure. *Bulletin of Environmental Contamination and Toxicology* 61: 169-174.

OECD (1998). *Daphnia magna* reproduction test - Test guideline 211. Organization for the Economic Cooperation and Development, Paris.

OECD (2000). *Daphnia* sp., acute immobilisation test. Revisal proposed for updating guideline 202. Organization for the Economic Cooperation and Development, Paris.

Orme S & Kegley S (2006). PAN pesticides database. Pesticide action Network, North America (San Francisco, CA 2006), http:www.pesticideinfo.org.

Pereira JL, Marques CR & Gonçalves F (2004). Allometric relations for *Ceriodaphnia* spp. and *Daphnia* spp. *Annales de Limnologie – International Journal of Limnology* 40: 11-14.

Pereira T, Cerejeira MJ & Espírito-Santo J (2000). Use of microbiotests to compare the toxicity of water samples fortified with active ingredients and formulated pesticides. *Environmental Toxicology* 15: 401-405.

Perera A, Burleigh JR & Davis CB (1999). Movement and retention of propanil *N*-(3,4-dichlorophenyl)propanamide in a paddy-riverine wetland system in Sri lanka. *Agriculture, Ecosystems and Environment* 72: 255-263.

Petrusek A, Bastiansen F & Schwenk K (2005). European *Daphnia* Species (EDS) - Taxonomic and genetic keys. [Build 2006-01-12 beta]. CD-ROM, distributed by the authors. Department of Ecology and Evolution, J.W. Goethe-University, Frankfurt am Main & Department of Ecology, Charles University, Prague.

Pieters BJ & Liess M (2006). Maternal nutritional state determines the sensitivity of *Daphnia magna* to short-term Fenvalerate exposure. *Aquatic Toxicology* 76: 268-277.

Polishchuk LV & Vijverberg J (2005). Contribution analysis of body mass dynamics in *Daphnia*. *Oecologia* 144: 268-277.

Quinn GP & Keough MJ (2002). Experimental design and data analysis for biologists. Cambridge University Press, Cambridge. 537p.

Smolders R, Bailieul M & Blust R (2005). Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. *Aquatic Toxicology* 73: 155-170.

Soares AMVM, Baird DJ & Callow P (1992). Interclonal variation in the performance of *Daphnia magna* Straus in chronic bioassays. *Environmental Toxicology and Chemistry* 11: 1477-1483.

Sobral O, Chastinet C, Nogueira A, Soares AMVM, Gonçalves F & Ribeiro R (2001). *In vitro* development of parthenogenetic eggs: a fast ecotoxicity test with *Daphnia magna*. *Ecotoxicology and Environmental Safety* 50: 174-179.

Stein JR (1973). Handbook of Phycological methods – Culture methods and growth measurements. Cambridge University Press, Cambridge. 448p.

Trubetskova I & Lampert W (1995). Egg size and egg mass of *Daphnia magna*: response to food availability. *Hydrobiologia* 307: 139-145.

Trubetskova I & Lampert W (2002). The juvenile growth rate of *Daphnia*: a short-term alternative to measuring the Per Capita Rate of Increase in Ecotoxicology. *Archives of Environmental Contamination and Toxicology* 42: 193-198.

Villarroel MJ, Sancho S, Ferrando MD & Andreu E (2003). Acute, chronic and sub-lethal effects of the herbicide propanil on *Daphnia magna*. *Chemosphere* 53(8), 857-864.

WHO 2004a. WHO Recommended classification of pesticides by hazard and guidelines to classification. World Health Organization, Geneva.

WHO 2004b. Chemical hazards in drinking water – Propanil in drinking water, WHO guidelines for drinking water quality (June 2006: http:// www.who.int/water_sanitation_health/dwq/chemicals/propanil/en/print.html). World Health Organization, Geneva.

Zar JH (1996). Biostatistical Analysis, 3rd Ed. Prentice-Hall, Inc. Prentice Hall: Upper Saddle River.

Capítulo IV

Efeitos da disponibilidade de alimento na toxicidade do insecticida Metomil em *Daphnia* spp.

NOTA PRÉVIA: O capítulo IV corresponde a um artigo científico que se encontra publicado numa revista internacional com arbitragem científica [*The Science of the Total Environment* 386: 9-20 (2007)]. Encontra-se, portanto, inteiramente escrito em língua inglesa, pelo que previamente se apresenta um resumo traduzido do trabalho constante do capítulo, acompanhado de uma lista de palavras-chave que a ele estão associadas.

Resumo | A utilização generalizada de pesticidas na produção agrícola constitui uma potencial ameaça à integridade dos ecossistemas de água doce estabelecidos nas imediações do local de aplicação, dado que frequentemente os seus resíduos contaminam o compartimento aquático. Dado o papel central dos zooplanctontes filtradores na cadeia alimentar, tais como *Daphnia*, as suas respostas a este tipo de contaminação proporciona informação relevante na estimativa dos riscos gerais que os pesticidas representam para o ecossistema aquático. Por outro lado, os cladóceros estão frequentemente sujeitos a flutuações ao nível da disponibilidade de recursos alimentares devido à dinâmica natural das comunidades fitoplanctónicas nos sistemas de água doce, sendo que a disponibilidade e a capacidade de aquisição alimentar condiciona o *fitness* geral das populações destes organismos. No presente estudo foram avaliadas as respostas de *Daphnia magna* e de três genótipos distintos pertencentes ao complexo *Daphnia longispina* a exposições agudas e crónicas do insecticida Metomil. Adicionalmente, verificou-se até que ponto as respostas sub-letais, individuais e populacionais de *Daphnia* ao insecticida podem ser moldadas pela concentração de alimento disponível. Os resultados demonstraram que o Metomil é um composto capaz de induzir efeitos tóxicos a concentrações muito reduzidas, quer na população de *D. magna* quer nas populações de *D.* cf *longispina*. Observaram-se ainda diferenças consideráveis no nível de sensibilidade, quando comparando as respostas ao tóxico dadas pelos diferentes *taxa*/genótipos. Mais ainda, a disponibilidade de alimento condicionou o *fitness* geral das populações, apesar de não ter sido possível detectar claramente contribuições específicas deste factor na resposta ao estímulo tóxico.

Palavras-chave | *Daphnia magna*; *Daphnia* cf *longispina*; Metomil; quantidade de alimento; respostas populacionais.

Effects of food availability on the acute and chronic toxicity of the insecticide Methomyl to *Daphnia* spp.

Joana Luísa Pereira & Fernando Gonçalves

CESAM& Departamento de Biologia da Universidade de Aveiro, 3810-193 Aveiro, Portugal.

Abstract | The widespread increase of pesticides application in crops threats vicinal freshwater lentic ecosystems, frequently leading to their contamination. Due to their position in the aquatic food web, the responses to these pesticide inputs of freshwater filter-feeding zooplankters, as daphnids, provide relevant information the general risk to the ecosystem of these xenobiotics. Moreover, cladoceran grazers often face fluctuations in food availability due to the phytoplankton dynamics in lentic water bodies, and food acquisition naturally conditions the fitness of these organisms. In this study, the responses of *Daphnia magna*, and of three genotypes within the *Daphnia longispina* complex, to acute and chronic exposures of Methomyl, were assessed. In addition, we focused on whether the food-level can model the *Daphnia* life-history responses to the insecticide. Results showed that Methomyl was acutely and chronically toxic to both *D. magna* and the *D.* cf *longispina* populations at very low concentrations, and remarkable differences in sensitivity were noticed when comparing the responses to the toxic among *taxa*/genotypes. Furthermore, food availability conditioned the overall fitness of the species although not interacting specifically on the response to the toxicant stress.

Key-words| *Daphnia magna*; *Daphnia* cf *longispina*; Methomyl; food-level; life-history.

INTRODUCTION

Pesticides are commonly used worldwide to face the need for increasing or improving agricultural production. There is a general trend to the development and preferable use of the so-called “benign pesticides” i.e. those pesticides which are selectively toxic, do not bioaccumulate, and have a relatively short persistence in the environment (Stark & Walter 1995). In this way, insecticidal products such as carbamates have gained favour especially when compared e.g. with organochlorine compounds (Hutson & Roberts 1985).

Methomyl [IUPAC: S-methyl N-(methylcarbamoyloxy)thioacetimidate] is a commonly used monomethyl carbamate insecticide, widely marketed since 1967 under the trade “Lannate” in certain fruit crops, vegetables, ornamentals and field crops (Tomlin 2001), to control a wide range of insects and spider mites through direct contact and ingestion (WHO 1996). It is a highly water soluble

compound (25ºC: 54.7 g/L), stable in distilled water for 30 d at a 5-7 pH, and has a low octanol:water partition coefficient (Kow = 1.24); this insecticide was found to adsorb poorly even to soil organic matter and its therefore expected to be mobile in soil; and, in field conditions, Methomyl does not get more than 20-30 cm into soil, which generally excludes ground water contamination by the chemical (WHO 1996). Contamination of water bodies via terrestrial runoff or leaching should be a rare event; however, since application procedures involve mainly ground or aerial spraying, surface water bodies in the crop vicinities can be directly contaminated.

The contamination of the aquatic ecosystem by Methomyl, as well as by other insecticides, is especially of concern when considering organisms inhabiting or having home ranges close to the surface layer of the water column: when reaching the water body, insecticides usually do not get instantaneously mixed in the water column; mixing occurs slowly, accompanied with the natural degradation and dissipation of the chemical (Van Wijngaarden et al. 2005). Moreover, the risk of such a contamination event increases when these non-target organisms have similar toxicant receptors as the target ones. WHO (1996, 2004) reports Methomyl as highly hazardous, and ranges it as moderately to highly toxic to several aquatic non-target organisms, highlighting *Daphnia magna* as very sensitive to the chemical: Methomyl registered a 48h-LC_{50} of 32µg/L and a chronic maximum acceptable toxicant concentration (MATC) in the range of 1.6-3.5µg/L for the species.

The particular mode of action of carbamates conditions their toxicity to non-target organisms such as *Daphnia*. They inhibit reversibly cholinesterase through the carbamylation of the enzyme and therefore it would be unlikely that single exposures induce long-lasting effects in surviving animals. Nevertheless, organisms inhabiting freshwater systems standing in agricultural areas often deal with frequent pesticide inputs resulting from repeated applications, and thus effects other than mortality (e.g. reproduction impairments) are likely to occur. Effects on reproduction or population growth of zooplankters, such as *Daphnia*, can be of a relevant ecological meaning since these organisms occupy a central position in the freshwater food web, and have a pivotal role as engagers of the energy transfer from producers to higher levels (Hanazato 1998).

Amongst other natural stressors, predatory pressure, intra and inter-specific interactions, and food availability can act singly or interactively to induce plasticity in *Daphnia* life-history traits (e.g. Gliwicz & Guisande 1992, Burns 2000, Pijanowska et al. 2006). Moreover, some studies have demonstrated that these natural stressors can modulate the effects of pesticides in *Daphnia*, and this joint action is likely to induce deleterious indirect effects at the population-, the community- and the

ecosystem-levels (see the reviews by Hanazato 2001 and by Relyea & Hoverman 2006). Within this context, food availability has a particular relevance since (i) phytoplankton densities in lentic ecosystems often fluctuate, and filter-feeding grazers need to deal with it by changing their life-history performances to optimally allocate the available resources (e.g. Gliwicz & Guisande 1992, Guisande & Gliwicz 1992, Boersma 1997); (ii) coping with physiological stress (as that promoted by pesticides) is energetically costly to daphnids (Smolders et al. 2005) - often inducing *per se* compensatory changes in their energetic metabolism (DeCoen & Janssen 2003, Smolders et al. 2005)-, and the nutritional state of the organism is a key factor conditioning this process (Heugens et al. 2001). Genotypic/species-specific differences were also shown to influence the *Daphnia* life-history responses to natural stressors (Kreutzer & Lampert 1999, Burns 2000, Antunes et al. 2003, Bernot et al. 2006, Gliwicz & Maszczyk 2007), to xenobiotics (Barata & Baird 1998, Antunes et al. 2004, Marques et al. 2004a,b), or to their joint action (Antunes et al. 2004, Pieters & Liess 2006, Pereira et al. 2007).

Regarding the role food availability as common natural stressor in freshwater ecosystems, and the potential ability of pesticides to harm non-target freshwater species, we intended to evaluate the combined effects of these stressors in survivorship and life-history of four different *taxa* belonging to the genus *Daphnia*. Previous studies have already reported more serious effects of a given toxic on *Daphnia* feeding in low food quantities, when compared with similar toxicant treatments having higher food supply (Folt et al. 1999, Pereira et al. 2007). This study was designed in order to assess how the toxicity of the insecticide Methomyl can be modelled by food, and whether this modelling pattern is consistent with those previously reported. Moreover, we focused on the relative differences in sensitiveness to acute and chronic Methomyl exposures of three distinct autochthonous genotypes belonging to the *D. longispina* complex; as well, the responses of these species were compared with those of the standard species *D. magna*. This analysis is expected to produce evidence on the relevance of the use of standard species in risk evaluation procedures.

MATERIAL & METHODS

Test organisms

The monoclonal *Daphnia* spp. bulk cultures were continuously reared in the lab, under a 16:8 hr light:dark photoperiod, at a temperature of 20±2ºC, in synthetic ASTM hardwater medium

(ASTM 1980) supplied with an organic additive extracted from the algae *Ascophyllum nodosum* (Baird et al. 1989). Cultures were renewed every other day and the organisms were fed with *Pseudokirchneriella subcapitata* at a rate of $3.0x10^5$ cells/ml for *Daphnia magna* (clone A, *sensu* Baird et al. 1989) and $1.5x10^5$ cells/ml for the *Daphnia* cf *longispina* clones. The three *D.* cf *longispina* clonal lineages used in tests were established from field-collected samples: (1) clone M was collected in lake Mira (Mira, centre-northwest of Portugal) and has been maintained in the lab since 2001 (clone EM7 *sensu* Antunes et al. 2003); (2) clone V resulted from a sample picked in lake Vela (Quiaios, centre-northwest of Portugal) in 2004; (3) clone T was collected in the shallow reservoir Tapada Grande (Mértola, southeast of Portugal) in 2004. Despite the species belonging to the subgenus *Hyalodaphnia* (commonly called *Daphnia longispina* group) are genetically well differentiated, interspecific hybridisation and backcrossing within the group often occurs (Schwenk & Spaak 1995, Schwenk et al. 2000). The high rates of interspecific hybridization, and the high degree of phenotypic plasticity observed in some traits traditionally seen as taxonomically discriminative, constrain appreciably a morphotype-based classification of the species/*taxa* within *Hyalodaphnia* (Schwenk et al. 2000, Billiones et al. 2004). ITS-RFLP techniques (Billiones et al. 2004) confirmed that these clones represent three distinct *taxa* within the *D. longispina* complex (Petrusek et al. 2005): although all the populations morphologically resemble *D. longispina* (according to Alonso 1996), the genotypes M, V and T are consistent with ITS-RFLP patterns and 12S DNA sequences of *D. galeata* x *longispina*, *D. longispina* x *galeata* and *D. galeata*, respectively. Hereinafter, we will refer to the different genotypes as *D. longispina* M, V and T for text clarity convenience.

Test procedures

The stock solution of the insecticide Methomyl was obtained by direct dilution of its commercial formulation Lannate® - 200 g/L Methomyl concentrated (Sapec Agro®, Portugal) - in distilled water, and was stored at 4ºC in dark. All the test solutions were freshly prepared by diluting the appropriate volumes of the stock solution in ASTM hardwater. Acute and life-history assays were carried independently for each *Daphnia* population in general accordance with respective standardized protocols (OECD 1998, 2000) and under the incubation conditions previously described for cultures. Seeking the reduction of maternal effects, only neonates born between the third and the fifth broods, no older than 24 hrs, were used in tests (mean body size of daphnids at the start of the experiments: 1.18, 0.70, 0.71, and 0.71mm for *D. magna*, *D. longispina* M, *D. longispina* V, and *D. longispina* T, respectively).

A static approach along 48-hrs, consisting in the exposure of the *Daphnia* populations to increasing nominal concentrations of Methomyl was employed for each of the acute assays. Twenty newborns per treatment (four replicates with five animals each) were exposed in glass vessels (test solution volume: 100ml), without food or organic additive supply. Dissolved oxygen and pH were monitored at the beginning and the end of the tests for validation purposes. Tests were screened for immobilised individuals at 24- and 48-hr exposure period, and records were used for further EC_{50} calculations (see *Statistics*).

In the chronic experiments, daphnids were individually exposed in glass vessels (test solution volume: 50ml), along 21 days, to five nominal concentrations of Methomyl plus one ASTM negative control (10 replicates per treatment). Renewal occurred every other day to freshly prepared test solutions. The life-history experiments followed a bifactorial design, where the four populations (*D. magna* and *D. longispina* M, V and T) were exposed to Methomyl at three different daily food rations (i.e. *P. subcapitata* at low, middle and high level): $0.75x10^5$, $1.5x10^5$ and $3.0x10^5$ cells/ml for *D. magna*; $0.375x10^5$, $0.75x10^5$, $1.5x10^5$ cells/ml for the three *D.* cf *longispina* populations. Dissolved oxygen and pH were monitored along the test for validation purposes. Tests were checked daily for eventual mortality and progeny releasing. The body size of the females was estimated at the beginning and at the end of the test, by extrapolation from the moult exopodite length (Pereira et al. 2004a).The somatic growth (*g*) of the females was then calculated:

$g = [\ln(l_f) - \ln(l_0)] / \Delta t$ (day^{-1}), where l_f is the final body length (mm), l_0 is the initial body length (mm) and Δt is the time range (days). Size records obtained during the survival period of females which died within the exposure-period were not integrated in data analysis and statistical procedures; the related fecundity data were only considered when calculating the per capita rate of population increase (*r*), where fecundity data and the eventual mortality occurrence were integrated through the Euler-Lotka equation:

$1 = \Sigma\ e^{-r.x} . l_x . m_x$, where *r* stands for the per capita rate of population increase (day^{-1}), x for the age class (days), l_x for the probability of surviving to age x, and m_x for the fecundity at age x. Uncertainties were estimated according to the Jackknife technique (Meyer et al. 1986).

Statistics

Regarding each acute assay, Probit analysis (Finney 1971) was used to estimate the 48-hr EC_{50} immobilisation values with the respective 95% confidence limits. For each *Daphnia* population, a two-way analysis of variance (two-way ANOVA) was used to assess the significance of the effects of food-level and Methomyl concentration, as well as of their interaction, on each of the life-history endpoints and on the population growth estimate. Additionally, we addressed the strength of association of each factor to the ANOVA model, by calculating the partial Eta-Squared value for each analysed endpoint/parameter (Pierce et al. 2004). As highly significant interactions between factors were detected, a one-way ANOVA, followed by the Dunnet post-hoc test, was carried out to detect differences in Methomyl treatments relatively to the control, within each food-level (Quinn & Keough 2002). Following Vanni & Lampert (1992) a multiple regression approach was used to test the fit of fecundity, age at first reproduction and growth rate as predictor variables of *r*. A forward selection procedure was employed, allowing the extraction of the relative individual contributions of each predictor to the overall fit (relative increase in the adjusted coefficient of determination) (Quinn & Keough 2002).

RESULTS

Both the acute and the chronic assays fulfilled the validity criteria outlined by OECD standard protocols (OECD 1998, 2000). Values of dissolved oxygen and pH were fairly constant (7.6-8.1 and 7.5-8.3 mg/L, respectively), and the observed slight fluctuations were not consistent with the increase of the toxicant concentrations.

Considering the acute toxicity, *D. magna* was ca. three-fold more tolerant than any of the *D.* cf *longispina* populations, by presenting an EC_{50} of 24.17µg/L (FIGURE IV.1). The highly sensitive *D.* cf *longispina* populations registered EC_{50}s between 4 and 10 µg/L, and the very tight confidence intervals did not overlap - Methomyl was more acutely toxic to *D. longispina* M (EC_{50} = 4.71µg/L), and *D. longispina* T was the most tolerant population to the insecticide (EC_{50} = 9.78µg/L).

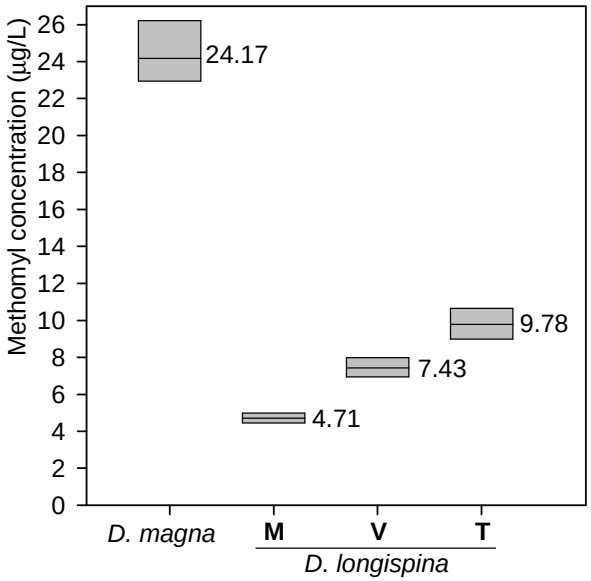


FIGURE IV.1 | Immobilisation 48-hr EC50 values (actual value specified next to each box - µg/L) and respective 95% confidence limits, for *D. magna* and for the three populations of *D.* cf *longispina*.

In all the tested populations, the treatment ranges used in the chronic assays felled bellow the EC_{50}s obtained in the acute toxicity screening. Despite some few exceptions, statistically significant effects were detected in all the assessed endpoints/parameters, as a response to increasing Methomyl doses. Moreover, mortality was low in all the chronic assays (<20%), and never compromised with the toxicant dose increase. The reproductive parameters and the growth rates were similarly impaired by Methomyl when comparing the responses between food levels: (i) statistics indicated a generally similar trend in the significance of the differences relatively to control, between food levels (TABLE IV.I; FIGURE IV.2 and IV.3); (ii) although, better overall life-history performances can be denoted as the food supply rises (FIGURE IV.2 and IV.3).

The inexistence of a statistically significant interaction between food and Methomyl treatments (TABLE IV.II) was often verified in several endpoints/parameters, except in fecundity and growth rate of *D. magna*, fecundity of *D. longispina* M, and growth rate of *D. longispina* T. Regarding the strength of association (partial Eta squared – TABLE IV.II), and where highly significant interactions were found, the ANOVA component "food" had a bigger contribution to the overall effects relatively to that of "methomyl"; in the remaining cases, "food" and "methomyl" interchanged in giving the higher contribution for the related effect. Despite the general lack of interaction between factors (see two-way ANOVA), the fecundity plots (FIGURE IV.2) show a slight trend for the smoothing of the response to increasing concentrations of Methomyl, with the decrease in food supply i.e. the stronger effects of the toxic seem to occur at the high food-level. The pattern of reduction in progeny

of *D. magna*, *D. longispina* M and *D. longispina* V with increasing toxicant concentrations, and the respective higher LOECs found in the lower food-level within *taxa*, can illustrate the described trend. This compensatory-like effect was not found in any of the remaining life-history endpoints/parameters, neither through the analysis of the curve steepness nor by direct assumptions based on LOEC values (FIGURE IV.3; TABLE IV.I).

TABLE IV.I: One-way ANOVA summary, and LOEC values (P≤0.05) (µg/L) obtained through the post-hoc Dunnet test, regarding the life-history responses of *Daphnia* to methomyl, within food-level (df – degrees of freedom).

	Endpoint	Food-level	df	$MS_{residual}$	F ratio	P value	LOEC
D. magna	Fecundity	low	5, 52	52.039	15.253	<0.001	9.5
		middle	5, 51	149.30	22.028	<0.001	14.2
		high	5, 48	137.74	35.030	<0.001	≤4.2
	Growth Rate	low	5, 52	3.44 e^{-7}	30.155	<0.001	≤4.2
		middle	5, 51	1.19 e^{-6}	11.589	<0.001	6.3
		high	5, 48	1.13 e^{-6}	18.448	<0.001	14.2
	Age at first reproduction	low	5, 52	0.4100	4.0800	0.003	14.2
		middle	5, 50	0.2100	7.1260	<0.001	14.2
		high	5, 48	0.2400	3.1880	0.0150	21.3
	r	low	5, 54	2.43 e^{-4}	22.412	<0.001	9.5
		middle	5, 54	5.85 e^{-4}	17.105	<0.001	14.2
		high	5, 54	4.27 e^{-4}	13.239	<0.001	14.2
***D. longispina* M**	Fecundity	low	5, 50	68.130	4.1750	0.003	2.8
		middle	5, 48	50. 190	25.321	<0.001	1.9
		high	5, 47	49.244	34.888	<0.001	≤0.8
	Growth Rate	low	5, 50	2.78 e^{-6}	10.720	<0.001	1.3
		middle	5, 48	2.25 e^{-6}	9.2400	<0.001	1.3
		high	5, 47	2.33 e^{-6}	10.207	<0.001	1.9
	Age at first reproduction	low	5, 50	0.4470	4.6920	0.001	4.2
		middle	5, 48	0.4770	0.1560	0.977	>4.2
		high	5,47	0.3240	2.3190	0.058	>4.2
	r	low	5, 54	4.17 e^{-4}	19.609	<0.001	2.8
		middle	5, 54	0.0011	6.2400	<0.001	2.8
		high	5, 54	3.91 e^{-4}	23.893	<0.001	2.8
***D. longispina* V**	Fecundity	low	5, 49	55.318	4.3470	0.002	3.5
		middle	5, 52	110.88	3.2120	0.013	3.5
		high	5, 49	51.685	3.8480	0.005	2.4
	Growth Rate	low	5, 49	2.29 e^{-6}	7.8740	<0.001	2.4
		middle	5, 52	3.97 e^{-6}	13.420	<0.001	1.1
		high	5, 49	2.52 e^{-6}	16.762	<0.001	1.1
	Age at first reproduction	low	5, 49	0.5750	15.539	<0.001	3.5
		middle	5, 52	1.8500	4.4550	0.002	3.5
		high	5, 49	0.7510	1.9540	0.102	>3.5
	r	low	5, 54	7.38 e^{-4}	10.942	<0.001	3.5
		middle	5, 54	7.38 e^{-4}	6.7570	<0.001	3.5
		high	5, 54	0.0011	2.1470	0.074	>3.5
***D. longispina* T**	Fecundity	low	5, 49	78.271	3.6490	0.007	2.5
		middle	5, 48	147.89	2.8570	0.025	5.6
		high	5, 50	221.21	4.8940	0.001	3.7
	Growth Rate	low	5, 49	1.67 e^{-6}	23.984	<0.001	1.7
		middle	5, 48	4.29 e^{-6}	11.636	<0.001	2.5
		high	5, 50	2.83 e^{-6}	19.875	<0.001	1.7
	Age at first reproduction	low	5, 49	0.6000	18.235	<0.001	3.7
		middle	5, 48	0.3810	19.947	<0.001	5.6
		high	5, 50	0.5080	10.453	<0.001	5.6
	r	low	5, 54	6.45 e^{-4}	18.022	<0.001	3.7
		middle	5, 54	8.14 e^{-4}	10.211	<0.001	3.7
		high	5, 54	6.17 e^{-4}	10.746	<0.001	5.6

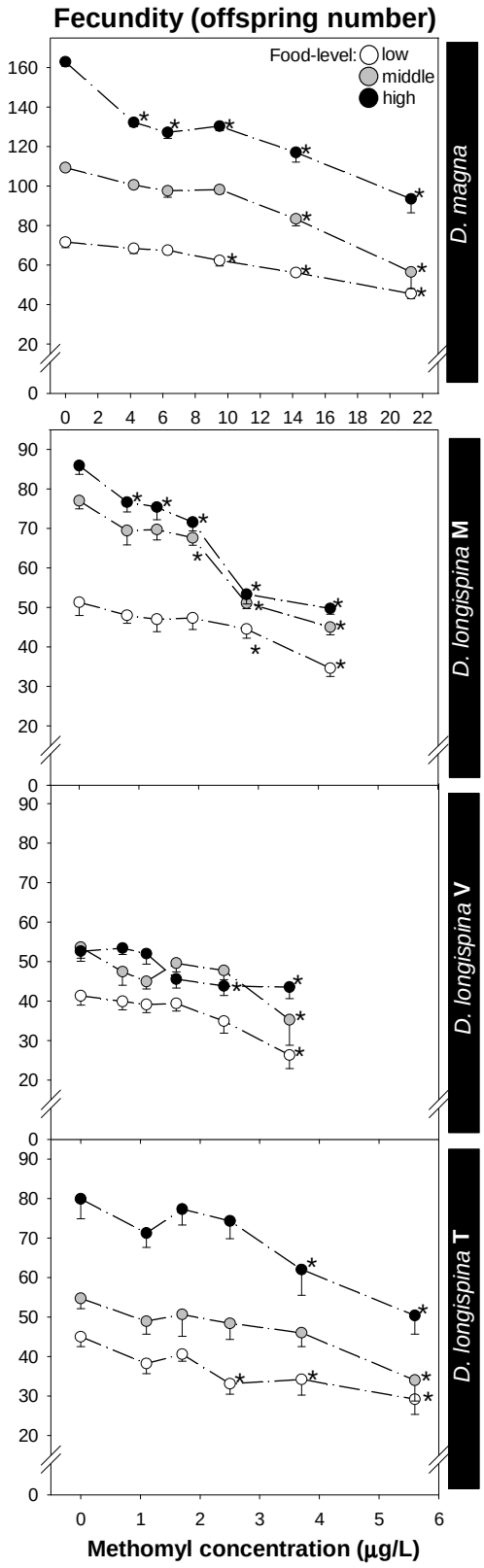


FIGURE IV.2 | Fecundity profiles (i.e. mean number of offspring yielded per female along the 21 days of the tests) for the four *taxa* along the Methomyl exposure at the different food-levels and associated significances relatively to the control (*) ($P \leq 0.05$). Error bars represent the standard error.

Within food-level, fecundity was always significantly impaired by very low Methomyl concentrations (TABLE IV.I; FIGURE IV.2), and *D. magna* was more tolerant to the insecticide than any of the *D.* cf *longispina* populations. When comparing among these latter, *D. longispina* M was generally the most sensitive population to the toxic – *D. longispina* T in the low food-level was the single exception to this trend, by presenting a LOEC of 2.5µg/L, slightly lower than the one registered for *D. longispina* M (2.8µg/L).

TABLE IV.II: Two-way ANOVA summary relative to the life-history responses of *Daphnia* exposed to Methomyl at different food-levels (df – degrees of freedom), with partial Eta-squared (partial η2) as a measure of the factors and interaction contribution to the overall effects.

	Endpoint	Source	df	MS	F ratio	P value	Partialη²
D. magna	Fecundity	Food	2, 151	6.15 e⁴	68.152	<0.001	0.879
		Methomyl	5, 151	7642.1	548.45	<0.001	0.693
		Food x Methomyl	10, 151	630.88	5.6260	<0.001	0.271
	Growth Rate	Food	2, 151	3.54 e⁴	406.32	<0.001	0.842
		Methomyl	5, 151	3.48 e⁵	39.581	<0.001	0.565
		Food x Methomyl	10, 151	4.96 e⁶	5.6170	<0.001	0.270
	Age at first reproduction	Food	2, 150	0.4090	1.4170	0.249	0.019
		Methomyl	5, 150	3.5900	12.433	<0.001	0.293
		Food x Methomyl	10, 150	0.1640	0.5690	0.837	0.037
	r	Food	2, 162	0.0298	71.157	<0.001	0.468
		Methomyl	5, 162	0.0198	47.207	<0.001	0.593
		Food x Methomyl	10, 162	6.79 e⁻⁴	1.6220	0.104	0.091
***D. longispina* M**	Fecundity	Food	2, 145	7510.0	133.94	<0.001	0.649
		Methomyl	5, 145	2734.2	48.765	<0.001	0.627
		Food x Methomyl	10, 145	276.53	4.9320	<0.001	0.254
	Growth Rate	Food	2, 145	3.36 e⁻⁴	136.82	<0.001	0.652
		Methomyl	5, 145	7.08 e⁻⁴	28.791	<0.001	0.498
		Food x Methomyl	10, 145	1.51 e⁻⁶	0.6140	0.800	0.040
	Age at first reproduction	Food	2, 145	2.9850	7.1570	0.001	0.090
		Methomyl	5, 145	1.9660	4.7140	<0.001	0.140
		Food x Methomyl	10, 145	0.4260	1.0200	0.429	0.066
	r	Food	2, 162	0.0119	18.626	<0.001	0.187
		Methomyl	5, 162	0.0229	35.727	<0.001	0.524
		Food x Methomyl	10, 162	7.86 e⁻⁴	1.2270	0.278	0.070
***D. longispina* V**	Fecundity	Food	2, 150	2110.3	28.754	<0.001	0.277
		Methomyl	5, 150	627.13	627.13	<0.001	0.222
		Food x Methomyl	10, 150	82.999	1.1310	0.343	0.070
	Growth Rate	Food	2, 150	2.22 e⁻⁵	7.5140	<0.001	0.091
		Methomyl	5, 150	1.04 e⁻⁴	35.319	<0.001	0.541
		Food x Methomyl	10, 150	3.78 e⁻⁶	1.2820	0.246	0.077
	Age at first reproduction	Food	2, 150	4.3860	4.0830	<0.001	0.052
		Methomyl	5, 150	15.680	14.598	0.019	0.329
		Food x Methomyl	10, 150	1.5230	1.4180	0.177	0.086
	r	Food	2, 162	0.0103	12.240	<0.001	0.131
		Methomyl	5, 162	0.0141	16.805	<0.001	0.342
		Food x Methomyl	10, 162	5.90 e⁻⁴	0.7010	0.722	0.041
***D. longispina* T**	Fecundity	Food	2, 147	1.51 e⁴	101.11	<0.001	0.579
		Methomyl	5, 147	1509.7	10.090	<0.001	0.256
		Food x Methomyl	10, 147	119.27	0.7970	0.632	0.051
	Growth Rate	Food	2, 147	4.94 e⁵	16.907	<0.001	0.187
		Methomyl	5, 147	1.33 e⁴	45.614	<0.001	0.607
		Food x Methomyl	10, 147	5.94 e⁶	2.0330	0.034	0.120
	Age at first reproduction	Food	2, 147	3.0980	6.2320	<0.001	0.078
		Methomyl	5, 147	22.510	45.274	0.003	0.606
		Food x Methomyl	10, 147	1.4840	1.4840	0.151	0.092
	r	Food	2, 162	0.0282	40.806	<0.001	0.335
		Methomyl	5, 162	0.0249	36.019	<0.001	0.526
		Food x Methomyl	10, 152	8.20 e⁻⁴	1.1850	0.304	0.068

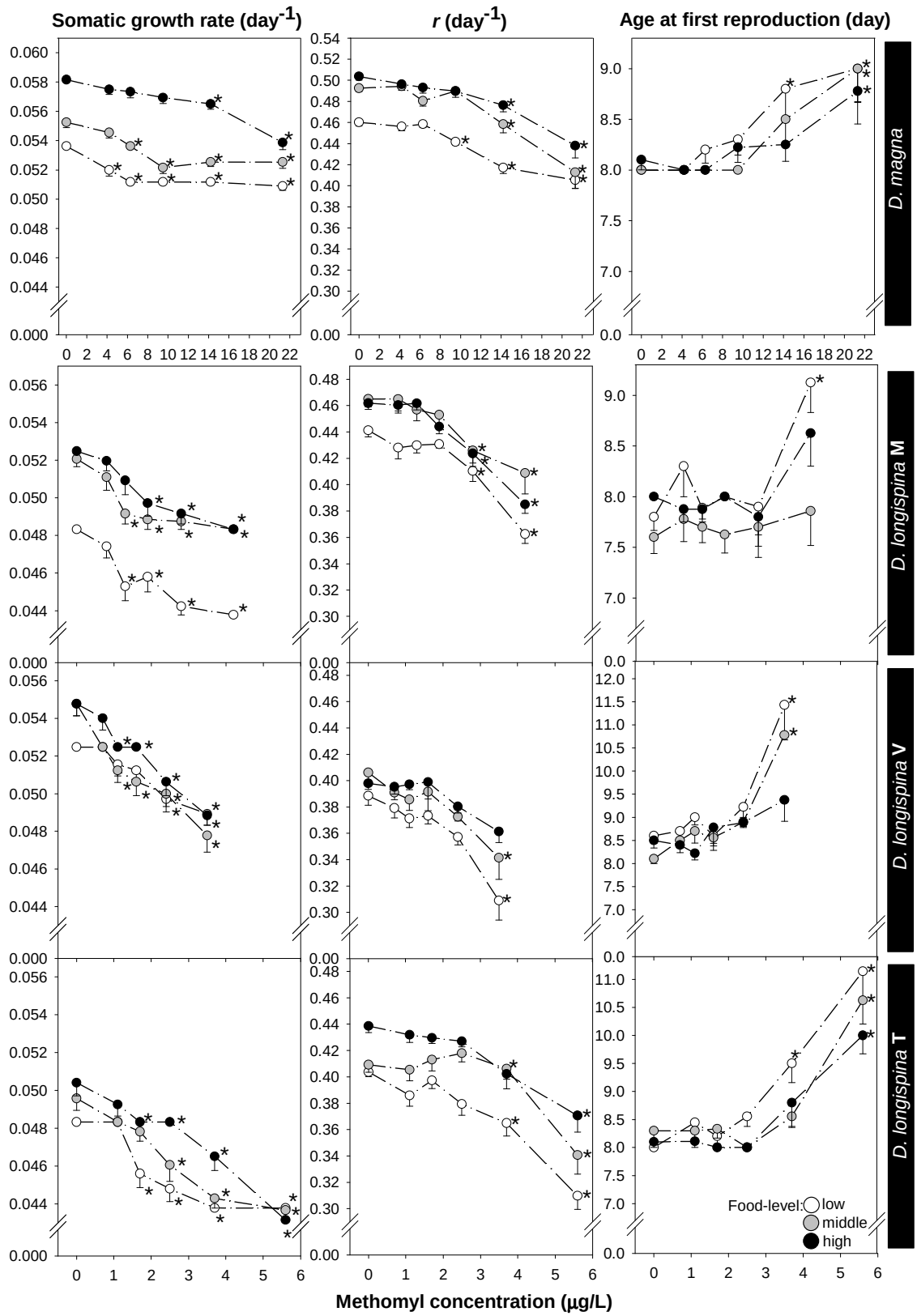


FIGURE IV.3 | Fecundity profiles (i.e. mean number of offspring yielded per female along the 21 days of the tests) for the four *taxa* along the Methomyl exposure at the different food-levels and associated significances relatively to the control (*) ($P \leq 0.05$). Error bars represent the standard error.

As well as in fecundity, the somatic growth rate and intrinsic rate of population increase (*r*) were significantly lowered by the insecticide concentrations, with *D. magna* presenting the higher tolerance among the tested *taxa* (TABLE IV.I; FIGURE IV.3). Statistics was quite consistent in detecting significant impairments of these parameters (Dunnet test; $P \leq 0.05$), when regarding the responses of the *D.* cf *longispina* populations within food-level, and even within *taxa* (FIGURE IV.3); *D. longispina* M was generally the most sensitive to Methomyl in all food scenarios (TABLE IV.I). The significant impairment of *r* with the toxicant concentration increase (irrespective the food-level) roughly follows the results obtained for fecundity (see above), and those regarding age at first reproduction (AFR). Despite with less responsiveness than fecundity, increasing Methomyl concentrations promoted a delay in reproduction, which was statistically significant for almost all species and food treatments (TABLE IV.I; FIGURE IV.3). Actually, when integrating fecundity, AFR and growth rate in a multiple regression approach using *r* as the dependent variable, AFR was consistently shown as the endpoint better explaining the model, and thus as the better contribution to changes in the population growth estimates (TABLE IV.III); there were a few cases where the growth rate explained enough variation to be also included in the model (*D. magna*, high food-level; *D. longispina* M, low and high food-level; *D. longispina* V, middle food-level; *D. longispina* T, high food-level), although this parameter was not directly used in the computation of *r*.

TABLE IV.III: Multiple stepwise regression of life-history endpoints [fecundity, age at first reproduction (AFR) and Growth Rate] on population growth rate; aR^2 represents the adjusted coefficient of determination for each entered independent variable in model.

	Food-level	**Variables entered (aR^2)**
D. magna	low	AFR (0.567); Fecundity (0.756)
	middle	AFR (0.766); Fecundity (0.894)
	high	AFR (0.643); Fecundity (0.833); Growth Rate (0.865)
***D. longispina* M**	low	AFR (0.641); Fecundity (0.780); Growth Rate (0.812)
	middle	AFR (0.436); Fecundity (0.721)
	high	AFR (0.441); Fecundity (0.740); Growth Rate (0.758)
***D. longispina* V**	low	AFR (0.793); Fecundity (0.849)
	middle	AFR (0.703); Fecundity (0.739); Growth Rate (0.760)
	high	AFR (0.511); Fecundity (0.540)
***D. longispina* T**	low	AFR (0.796); Fecundity (0.866)
	middle	AFR (0.728); Fecundity (0.804)
	high	AFR (0.742); Fecundity (0.816); Growth Rate (0.832)

Overall, *D. magna* was always more tolerant to the toxicant than the *D.* cf *longispina* populations. However, it seems that the differences in tolerance were lower in magnitude when regarding the chronic exposures relatively to the acute ones. The pattern of sensitivity among *D.* cf *longispina* populations obtained in the acute exposures was not maintained from the acute to the chronic exposures - if fecundity and *r* denoted *D. longispina* M as the most sensitive population to the toxic in the chronic assays, irrespective the food level, the somatic growth and AFR did not confirmed this trend.

DISCUSSION

Methomyl is a widely used carbamate insecticide due to the advantages resulting from its high insecticidal activity with rapid reversibility, and its relative low persistence when compared with other insecticidal classes (Hutson & Roberts 1985). This insecticide has been fairly studied in terms of its efficiency in controlling target pests (e.g. Reitz et al. 1999, Ehler 2004); however, and considering the non ecologically-selective profile of methomyl (Ehler 2004), its toxicity to aquatic non-target organisms and the further consequences for the aquatic ecosystem need better attention.

The results confirmed the high acute toxicity of methomyl for *Daphnia* spp.. The 48-h EC_{50} that we found for *D. magna* (24.17µg/L) was slightly lower than those available in literature: by using technical grade Methomyl, WHO (1996) reports a 48h-LC_{50} of 32µg/L, while Tomlin (2001) found a very similar value for the same endpoint (31.7µg/L), and Fernández-Alba et al. (2002) refer to a 48h-EC_{50} of 28.7µg/L. These differences are probably due to different culture methods or distinct genotypes used for the experiments, rather than to differences in the purity of the Methomyl solution used. In fact, some adjuvant chemicals used in the formulation of the commercial solutions of pesticides were already shown to be toxic to *Daphnia* species (e.g. Stark & Walthall 2003). However, previous experiments carried in our lab denoted *D. magna* as slightly more tolerant to the commercial formulation of Methomyl (Lannate®) than to the technical grade chemical (unpublished data). As far as we could notice, there is a single register regarding the acute toxicity of the insecticide to smaller *Daphnia* species, defining a LC_{50} for *D. longispina* of 220µg/L (Orme & Kegley 2006); this value is, however, completely out of the toxicity range that we obtained in our study. The relative higher tolerance of *D. magna* to the toxic was expected since the smaller size of *D.* cf *longispina*, with the consequent greater surface-to-volume ratio, would expose more the organism to the waterborne chemical; and, in the absence of food, the main intake route of a toxicant would be

through contact with body surface (Vesela & Vijverberg 2007). This pattern of sensitivity has actually been confirmed by other authors comparatively testing the effect of organic chemicals in *D. magna* and *D. longispina* (Antunes et al. 2004, Marques et al. 2004a,b).

The life-history traits and population growth of the *Daphnia* populations used in this study were impaired by Methomyl i.e. regardless the food constraints, daphnids lowered their fitness as the toxicant concentrations increased. Pereira et al. (2007) compared the effects of a toxicant stress in *Daphnia* spp. feeding in different food-quantity levels, and found consistent evidences of a higher toxicant resistance of the organisms tested at lower food-levels. Folt et al. (1999) reported the same pattern when regarding the combined effect of low food supply and an organic surfactant toxicant i.e. they found that *Daphnia* withstand better than what was predicted by the multiple stressor models applied. In this study, the lower impact of the toxicant in low food supply conditions was related with its lower intake via filtering water and consuming toxicant-rich food. Methomyl is, however, a contact insecticide (Tomlin 2001) and its main intake route is likely to be through body surface rather than via filtration-related mechanisms. Although some trends arise from a direct graphical analysis, the mentioned compensatory-like effects cannot be confirmed in the present study since statistics only depicted few interactions between food and methomyl as factors in the two-way ANOVA procedures. Even though, food actually modeled the effects of the toxic: (i) the higher the food-level the better the overall fitness; (ii) irrespective the species or the food-level, food was frequently shown to be the factor contributing the most to explain variance. Daphnids cope with fluctuating levels of food availability by adjusting the allocation of their actual energy budget. As food resources depress, they would disinvest in growth and reproduction, and thus smaller females will release smaller broods of larger, e.g. more hunger-resistant offspring (Gliwicz & Guisande 1992, Trubetskova & Lampert 1995, Boersma 1997). If well-fed *Daphnia* may allocate a great part of their energy budget to reproduction, the depression of food conditions will promote a shift in allocation towards self-maintenance, to increase longevity and lifetime reproduction (Smolders et al. 2005). This investment in self-maintenance is likely to increase stress-resistance given that more energy is available to cope with an additional stressor (Smolders et al. 2005, Polishchuk & Vijverberg, 2005). The somatic growth was similarly affected in all the food-levels and reproduction seems to be less impaired in deficiently-fed *Daphnia* populations. This suggests that our low food-level was not low enough to promote a relevant shift in the resources allocation towards self-maintenance, and thus a better ability to cope with methomyl stress in low-food environments was not clear in our populations. On the other hand, our pattern of toxicant effects under limiting food conditions is in agreement with several other

investigations (see the review by Heugens et al. 2001); a low nutritional supply can increase *Daphnia* sensitivity to stress given that fewer resources are available for use in physiological detoxification mechanisms.

The per capita rate of population increase (*r*) does not allow direct predictions for field conditions since its computation does not include important effects of natural stressors featuring the performance of natural populations in the field [e.g. density-dependent effects conditioning intraspecific interaction/competition (Forbes et al. 2001, Liess 2002)]. Still, *r* provides a more ecologically relevant measure of effects than a single endpoint such as reproduction or mortality (Forbes & Calow 1999), giving a more reliable results summary regarding ecological impact evaluations and risk assessment requirements (Forbes & Calow 1999, Jager et al. 2004). In the present study, changes in the population growth were always found to be fundamentally driven by methomyl increasing concentrations rather than by food availability (see Table IV.II); and, *r* was significantly impaired by very low concentrations of Methomyl (specially in *D.* cf *longispina* populations), irrespective of the *taxon*. Wilson & Foos (2006) found Methomyl concentrations slightly below our LOEC's for *r* in surface water samples, and still concluded that the insecticide can contribute significantly acute risks to freshwater organisms. Moreover, the MATC established by WHO to methomyl ranges between 1.6 and 3.5 µg/L (WHO, 1996), which in face of our data underestimates risks, in particular to *D. longispina* populations. This corroborates our concern on the ability of methomyl to structurally affect *Daphnia* natural populations. In agreement with literature (e.g. Vanni & Lampert 1992), our results additionally show that significant impairments of *r* were mainly driven by a delay in reproduction, although age at first reproduction showed the less direct responsiveness when compared with the other assessed single-endpoints. In fact, slight delays in reproduction can have important consequences in rapidly expanding *Daphnia* natural populations, given that (i) the net offspring output during the female lifespan can be remarkably reduced, which compromises the overall population fitness; (ii) under natural conditions daphnids often mature earlier at smaller sizes to minimize size-selective predation (e.g. Lampert 1991, Tessier et al. 1992), and when delays in first reproduction occur, larger females will more easily be predated. In addition, when maturation is retarded, the juvenile cohorts will prevail for longer; and considering that this was proven to be the *Daphnia* life-stage the most sensitive to toxicants (Hanazato 2001), the population would be more susceptible to a further acting toxicant stress.

Following the trend observed in the acute exposures, Methomyl was chronically more toxic to the *D.* cf *longispina*_populations than to *D. magna*, irrespective of food-level. Considering that Methomyl is a contact insecticide (e.g. Tomlin, 2001), the main intake route of the toxicant in *Daphnia* will be mostly through body surface rather than via filtration of toxicant-bound food particles (see Allen et al. 1995 for considerations on this latter issue). Accordingly, a lower surface-to-volume ratio may explain the lower sensitivity to Methomyl of *D. magna*. As to the *D.* cf *longispina* populations, one can not argue differences in the surface-to-volume ratio to explain different Methomyl acute and chronic toxicity, since their body size is very similar; a one-way ANOVA approach was used to compare the adult body size of the *D.* cf *longispina* females of the control treatments (using the three control treatments within each *taxa* pooled as a single group), and statistically significant differences between *taxa* were not found concerning this trait, neither when considering the primipara size ($F = 2.221$; $df = 2, 87$; $P = 0.115$) nor when regarding the size of the females after 21 days ($F = 2.288$; $df = 2, 87$; $P = 0.108$). The three *D.* cf *longispina* populations were found to be genetically distinct, and therefore their tolerance to the toxicant can be constrained by the genotype. These clonal lineages were isolated from distinct and geographically isolated shallow systems, which face particular pollution- and nutrient loading scenarios (Castro et al. 2005, Abrantes 2006, Pereira et al. 2004b). Thus, it is likely that these particular environmental conditions constrain the *Daphnia* population dynamics and the prevailing genotypes. The acute tests demarcated *D. longispina* M as the most sensitive population to Methomyl, and this trend was confirmed by fecundity and *r* in the life-history assays, irrespective of food-level. Further inferences on the relative sensitivity of the *D.* cf *longispina* populations to the toxic can not be advanced since no clear patterns can be extracted regarding the remaining life-history endpoints and accounting to the different food scenarios. These considerations are conditioned by the shortcomings of the NOEC-LOEC approach on the clarification of threshold concentrations of toxicants in the environment (e.g. Van der Hoeven 1997). However, this work focused the relative toxicity of Methomyl in different food scenarios, and in this way it should be noticed that different species/*taxa* had relevant differences in between, when regarding lethal and sub-lethal endpoints. In fact, differences between species in tolerance have been shown to be of a critical influence in driving structural and functional adjustments as a response to stress, in natural populations and communities (Hanazato 1998, 2001).

CONCLUSION

Pesticide effects in zooplankton were already shown to be conditioned by several natural stressors, including predatory pressure, intra and inter-specific interactions, and food availability (e.g. Hanazato 1998, 2001, Heugens et al. 2001, Relyea & Hoverman 2006). Our findings confirmed the ability of food in modelling the response of four *Daphnia* populations to a carbamate insecticide; in particular, we observed an increasing sensitivity of the organisms to the toxicant stress with the decrease of food supply. Experiments regarding the toxicity of pesticides under different environmental conditions can indeed generate valuable data on the estimation of the chemicals' hazardous potential, and should therefore be considered when designing procedures under the scope of environmental risk assessment programs. Furthermore, this study also provides evidences for discussion on the use of *D. magna* as a standard species for regulatory purposes or integrated in ecologic risk assessment programs, suggesting that data can be compromised in reliability, particularly when this species is not directly related with (e.g. does not inhabit) the studied system.

REFERENCES

Abrantes N, Pereira R & Gonçalves F (2006). First step for an ecological risk assessment to evaluate the impact of diffuse pollution in lake Vela (Portugal). *Environmental Monitoring and Assessment* 117: 411-431.

Allen Y, Calow P & Baird DJ (1995). A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. *Environmental Toxicology and Chemistry* 14: 1625-1630.

Alonso M (1996). Crustacea – Branchiopoda. *In:* MAR Sánchez (coord. ed.), Fauna Iberica, Vol. 7. Museo Nacional de Ciencias Naturales, CSIC, Madrid. 486p.

Antunes SC, Castro BB & Gonçalves F (2003). Chronic responses of different clones of *Daphnia longispina* (field an ephippia) to different food-levels. *Acta Oecologica* 24: S325-S332.

Antunes SC, Castro BB & Gonçalves F (2004). Effect of food level on the acute and chronic responses of daphnids to lindane. *Environmental Pollution* 127: 367-375.

ASTM (1980). Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Report E 729-80. American Society for Testing and Materials, Philadelphia.

Baird DJ, Soares AMVM, Girling A, Barber I, Bradley MC & Calow P (1989). The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. *In*: H Lokke, H Tyle & F Bro-Rasmussen (eds.). Proceedings of the First European Conference on Ecotoxicology, Lyngby, Denmark, pp 144-148.

Bernot RJ, Dodds WK, Quist MC & Guy CS (2006). Temperature and kairomone induced life history plasticity in coexisting *Daphnia*. *Aquatic Ecology* 40: 361-372.

Boersma M (1997). Offspring size and parental fitness in *Daphnia magna*. *Evolutionary Ecology* 11: 439-450.

Burns CW (2000). Crowding-induced changes in growth, reproduction and morphology of *Daphnia*. *Freshwater Biology* 43: 19-29.

Billiones R, Brehm M, Klee J & Schwenk K (2004). Genetic identification of *Hyalodaphnia* species. *Hydrobiologia* 526: 43-53.

Barata C & Baird DJ (1998). Phenotypic plasticity and constancy of life-history traits in laboratory clones of *Daphnia magna* Straus: effects of neonatal length. *Functional Ecology* 12: 442-452.

Castro BB, Antunes SC, Pereira R, Soares AMVM & Gonçalves F (2006). Rotifer community structure in three shallow lakes: seasonal fluctuations and explanatory factors. *Hydrobiologia* 543: 221-232.

De Coen WM & Janssen CR (2003). The missing biomarker link: relationship between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental Toxicology and Chemistry* 22: 1632-1641.

Ehler LE (2004). An evaluation of some natural enemies of *Spodoptera exigua* on sugarbeet in Northern California. *Biocontrol* 49: 121-135.

Fernández-Alba AR, Hernando D, Agüera A, Cáceres J & Malato S (2002). Toxicity assays: a way for evaluating AOPs efficiency. *Water Research* 36: 4255-4262.

Finney DJ (1971). Probit analysis. Cambridge University Press, Cambridge. 350 p.

Folt CL, Chen CY, Moore MV & Burnaford J (1999). Synergism and antagonism among multiple stressors. *Limnology and Oceanography* 44(3 part2): 864-877.

Forbes VE & Calow P (1999). Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? *Environmental Toxicology and Chemistry* 18: 1544-1556.

Forbes VE, Sibly RM & Calow P (2001). Toxicant impacts on density-limited populations: a critical review for theory, practice, and results. *Ecological Applications* 11: 1249-1257.

Gliwicz ZM & Guisande C (1992). Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia* 91: 463-467.

Gliwicz ZM & Masczyk P (2007). *Daphnia* growth is hindered by chemical information on predation risk at high but not at low food levels. *Oecologia* 150: 706-715.

Guisande C & Gliwicz ZM (1992). Egg size and clutch size in two *Daphnia* species grown at different food-levels. *Journal of Plankton Research* 14: 997-1007.

Hanazato T (1998). Response of a zooplankton community to insecticide application in experimental ponds: a review and the implications of the effects of chemicals on the structure and functioning of freshwater communities. *Environmental Pollution* 101: 361-373.

Hanazato T (2001). Pesticide effects on freshwater zooplankton: an ecological perspective. *Environmental Pollution* 112: 1-10.

Heugens EHW, Hendriks AJ, Dekker T, Van Straalen N & Admiraal W (2001). A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. *Critical Review of Toxicolology* 31: 247-284.

Hutson DH & Roberts TR. Insecticides – Progress in Pesticide Biochemistry and Toxicology. *In*: DH Hutson & TR Roberts (Eds.), Insecticides. John Wiley & Sons, West Sussex. pp 1-34.

Jager T, Crommentuijn T, Van Gestel CAM & Kooijman SALM (2004). Simultaneous modelling of multiple end points in Life-Cycle toxicity tests. *Environmental Science & Technology* 38: 2894-2900.

Kreutzer & Lampert W (1999). Exploitative competition in differently sized *Daphnia* species: a mechanistic explanation. *Ecology* 80: 2348-2357.

Lampert W (1991). The dynamics of *Daphnia magna* in a shallow lake. *Internationale Vereinigung fur theorische und angewandte Limnologie, Verhandlungen* 24: 795-798.

Liess M (2002). Population response to toxicants is altered by intraspecific interaction. *Environmental Toxicology and Chemistry* 21: 138-142.

Marques CR, Abrantes N & Gonçalves F (2004a). Life-history traits of standard and autochtonous cladocerans: I. Acute and Chronic effects of acetylsalicylic acid. *Environmental Toxicology* 19: 518-526.

Marques CR, Abrantes N & Gonçalves F (2004b). Life-history traits of standard and autochtonous cladocerans: II. Acute and chronic effects of acetylsalicylic acid metabolites. *Environmental Toxicology* 19: 527-540.

Meyer JS, Ingersoll CG, McDonald LL & Boyce MS (1986). Estimating uncertainty in population growth rates: Jackknife vs. Bootstrap techniques. *Ecology* 67: 1156-1166.

OECD (1998). *Daphnia magna* reproduction test - Test guideline 211. Organization for the Economic Cooperation and Development. Paris.

OECD (2000). *Daphnia* sp., acute immobilisation test. Revisal proposed for updating guideline 202. Organization for the Economic Cooperation and Development. Paris.

Orme S & Kegley S (2006). PAN pesticides database. Pesticide action Network, San Francisco, http: www.pesticideinfo.org.

Pereira JL, Marques CR & Gonçalves F (2004a). Allometric relations for *Ceriodaphnia* spp. and *Daphnia* spp. *Annales de Limnologie – International Journal of Limnology* 40: 11-14.

Pereira JL, Mendes CD & Gonçalves F (2007). Short- and long-term responses of *Daphnia* spp. to Propanil exposures in distinct food supply scenarios. *Ecotoxicology and Environmental Safety* 68: 386-396.

Pereira R, Ribeiro R & Gonçalves F (2004b). Plan for an integrated Human and Environmental Risk Assessment in the S. Domingos mine area (Portugal). *Human and Ecological Risk Assessment* 10: 543-578.

Pierce CA, Block RA & Aguinis H (2004). Cautionary note on reporting eta-squared values from multifactor ANOVA designs. *Educational and Psychological Measurement* 64: 916-924.

Petrusek A, Bastiansen F & Schwenk K (2005). European *Daphnia* Species (EDS) - Taxonomic and genetic keys. [Build 2006-01-12 beta]. CD-ROM, distributed by the authors. Department of Ecology and Evolution, J.W. Goethe-University, Frankfurt am Main, Germany & Department of Ecology, Charles University, Prague.

Pieters BJ & Liess M (2006). Maternal nutritional state determines the sensitivity of *Daphnia magna* to short-term Fenvalerate exposure. *Aquatic Toxicology* 76: 268-277.

Pijanowska J, Dawidowicz P, Howe A & Weider LJ (2006). Predator-induced shifts in *Daphnia* life-histories under different food regimes. *Archives für Hydrobiologie* 167: 37-54.

Polishchuk LV & Vijverberg J (2005). Contribution analysis of body mass dynamics in *Daphnia*. *Oecologia* 144: 268-277.

Quinn GP& Keough MJ (2002). Experimental design and data analysis for biologists. Cambridge University Press, Cambridge. 537 p.

Reitz SR, Kund GS, Carson WG, Phillips PA & Trumble JT (1999). Economics of reducing insecticide use on celerity through low-input pest management strategies. *Agriculture, Ecosystems and Environment* 73: 185-197.

Relyea R & Hoverman J (2006). Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters* 9: 1157-1171.

Schwenk K & Spaak P (1995). Evolutionary and ecological consequences of interspecific hybridization in cladocerans. *Experientia* 51: 465-481.

Schwenk K, Posada D & Hebert PDN (2000). Molecular systematics of European Hyalodaphnia: the role of contemporary hybridization in ancient species. *Proceedings of the Royal Society of London B* 267: 1833-1842.

Smolders R, Bailieul M & Blust R (20059. Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. *Aquatic Toxicology* 73: 155-170.

Stark JD & Walter JF (1995). Neem oil components affect the efficacy of commercial neem insecticides. *Journal of Agriculture and Food Chemistry* 43: 507-512.

Stark JD & Walthall WK (2003). Agricultural adjuvants: acute mortality and effects on population growth rate of *Daphnia pulex* after chronic exposure. *Environmental Toxicology and Chemistry* 22: 3056-3061.

Tessier AJ, Young A & Leibold M (1992). Population dynamics and body-size selection in *Daphnia*. *Limnology and Oceanography* 37: 1-13.

Tomlin CDS (2001). *The pesticide manual*. British Crop Protection Council, Surrey.

Trubetskova I & Lampert W (1995). Egg size and egg mass of *Daphnia magna*: response to food availability. *Hydrobiologia* 307: 139-145.

Van der Hoeven N (1997). How to measure no effect. Part III: Statistical aspects of NOEC, ECx and NEC estimates. *Environmetrics* 8: 255-261.

Van Wijngaarden RPA, Brock TCM & Van Den Brink PJ (2005). Threshold levels for effects of insecticides in freshwater ecosystems: a review. *Ecotoxicology* 14: 355-380.

Vanni MJ & Lampert W (1992). Food quality effects on life-history traits and fitness in the generalist herbivore *Daphnia*. *Oecologia* 92: 48-57.

Vesela S & Vijverberg J (2007). Effect of body size on toxicity of zinc in neonates of four differently sized *Daphnia* species. *Aquatic Ecology* 41: 67-73.

WHO (1996). Methomyl – Environmental Health Criteria 178. World Health Organisation, Geneva.

WHO (2004). The WHO recommended classification of pesticides by hazard and guidelines to classification World Health Organisation, Geneva.

Wilson PC & Foos JF (2006). Survey of carbamate and organophosphorous pesticide export from a South Florida (USA) agricultural watershed: implications of sampling frequency on ecological risk estimation. *Environmental Toxicology and Chemistry* 25: 2847-2852.

Capítulo V

Expressão genética em *Daphnia magna*: efeitos de exposições agudas ao insecticida Metomil e ao herbicida Propanil.

NOTA PRÉVIA: O capítulo V corresponde a um artigo científico que se está em fase final de preparação e será brevemente submetido a uma revista internacional com arbitragem científica. Encontra-se, portanto, inteiramente escrito em língua inglesa, pelo que previamente se apresenta um resumo traduzido do trabalho constante do capítulo, acompanhado de uma lista de palavras-chave que a ele estão associadas.

Resumo | *Daphnia magna* é uma das espécies mais utilizadas na avaliação dos efeitos potenciais de diversos xenobióticos, tais como os pesticidas, em organismos aquáticos não-alvo. Este estudo centra-se na avaliação do potencial do insecticida carbamato Metomil e do herbicida acetanilídico Propanil para induzir efeitos fenotípicos deletérios e alterações da expressão genética em *D. magna*. O Metomil é um inibidor da Acetilcolinesterase bastante utilizado nas produções agrícolas para controlar uma larga gama de insectos patogénicos e o Propanil é largamente utilizado no período de pós-emergência do arroz para proteger a cultura de diversas pragas vegetais. Resíduos de ambos os pesticidas foram já detectados nos canais de drenagem ou em massas de água superficiais nas vizinhanças dos campos agrícolas. Os efeitos fenotípicos de cada químico individualmente foram avaliados através de um ensaio normalizado de imobilização. Os padrões de imobilização foram relacionados com os níveis gerais de expressão do mARN através da exposição aguda de *D. magna* aos 48h-EC_1s estimados previamente para cada um dos pesticidas, seguida de hibridização do mARN extraído após exposição com *microarray* de cADN, composto por 15000 clones representativos de mais de 5000 *ESTs* únicos. Esta ferramenta molecular mostrou perfis de expressão únicos para cada pesticida, considerando que, de forma a maximizar a reproducibilidade, só foram considerados na análise os *spots* (equivalentes aos *ESTs*) cuja resposta foi duas vezes superior e significativamente diferente do controlo (ANOVA de 1 via, P <0.05). Foi detectada expressão genética diferencial em 768 *spots*, com grande incidência de indução de genes em ambos os pesticidas. O metabolismo energético (e.g., proteínas mitocondriais e proteínas relacionadas com a síntese de ATP), a ecdise (e.g., proteínas receptoras de quitina e proteínas cuticulares) e a biossíntese de proteínas (e.g., proteínas ribossomais e factores de transcrição) foram os processos funcionais com maior destaque na expressão genética diferencial. Mais especificamente, o insecticida metomil induziu genes que codificam proteínas envolvidas em processos de homeostase iónica e metabolismo de xenobióticos, enquanto o herbicida propanil induziu a síntese de hemoglobina, bem como de proteínas relacionadas com mecanismos de defesa (e.g. sistemas de resposta imunitária inata) e relacionadas com vias de comunicação neuronal.

Palavras-chave | *Daphnia magna*; Metomil; Propanil; exposições agudas; *microarrays*; expressão genética.

Gene expression in *Daphnia magna*: effects of acute exposure to a carbamate insecticide and an acetanilide herbicide

Joana Luísa Pereira†, Christopher J Hill‡, Richard M Sibly‡, Viacheslav Bolshakov‡, Fernando Gonçalves†, Lars-Henrik Heckmann‡ & Amanda Callaghan‡

†CESAM& Departamento de Biologia da Universidade de Aveiro, 3810-193 Aveiro, Portugal.

‡Environmental Biology Section, School of Biological Sciences, The University of Reading, Whiteknights, PO Box 68, Reading RG6 6BX, United Kingdom.

Abstract | The water flea, *Daphnia magna*, is a widely used species in Ecotoxicology and is employed to measure the effects of xenobiotics, such as pesticides, on non-target aquatic organisms. In this study, we looked at the effect of the carbamate insecticide Methomyl and the acetanilide herbicide Propanil on *D. magna* life-history (survival) and gene expression. Methomyl is an acetylcholinesterase inhibitor broadly used to control a wide range of insects in agriculture, and Propanil is commonly used in the post-emergence of rice for protection against several grass and broad-leaf weeds. Both pesticides have been found in drainage ditches and surface water bodies standing in crop vicinities. The phenotypic effects of single doses of each chemical were evaluated using a standard immobilisation assay. Immobilisation was linked to global mRNA expression levels using the previously estimated 48h-EC_1s, followed by hybridisation to a cDNA microarray with 15000 clones representing >5000 unique ESTs. In order to maximise reproducibility, only spots (equivalent to ESTs), which responded >2-fold and were significantly (1-way ANOVA, $P < 0.05$) different from control were considered for analysis. Differential gene expression was found significant in 768 spots, and gene up-regulation was highly registered for both the tested chemicals. Microarray analysis revealed unique expression profiles for Methomyl and Propanil where energy metabolism (e.g. mitochondrial proteins, ATP synthesis-related proteins), moulting (e.g. chitin binding proteins, cuticular proteins) and protein biosynthesis (e.g. ribosomal proteins, transcription factors) were the biological processes assigning higher levels of differentially expressed sequences. Methomyl was able to induce the expression of genes coding for proteins involved in specific processes such as ion homeostasis and xenobiotics metabolism; conversely, Propanil seems to highly promote Haemoglobin synthesis and to up-regulate genes specifically related with defence mechanisms (e.g. innate immunity response systems) and neuronal pathways.

Key-words| *Daphnia magna*; Methomyl; Propanil; acute exposures; microarrays; gene expression.

INTRODUCTION

Significant amounts of pesticides are intentionally released into the environment to control destructive insects, weeds and pathogens. Surface water bodies, standing in or nearby agricultural areas, can contaminated with pesticides (Carter 2000), which generally occurs through spray drift during application, surface runoff and/or leaching (Brown et al. 1995, Flury 1996, Carter 2000, Huber et al. 2000, Reichenberger et al. 2007). Thus, non-target aquatic species are likely to be exposed to these xenobiotics, particularly if inhabiting or having home ranges close to the water surface given the general pattern of the pesticides mixing in the water column (Van Wijngaarden et al. 2005). Within freshwater organisms, *Daphnia* has been considered an ecologically relevant model due to its central position in the food web and pivotal role in structuring the pelagical trophic interactions (e.g., Lampert 2006). Daphnids are particularly suitable to be reared in lab and, as they are cyclical parthenogens, genetic variance can be highly controlled within and between experiments. In this way, and additionally considering its recognised high sensitivity to toxicant stress, *Daphnia* has been extensively used as a feasible model in ecotoxicological studies, such as those addressing effects of pesticides in non-target organisms (e.g., Hanazato 2001).

Even the so-called 'contemporary pesticides', which are selectively toxic, do not bioaccumulate, and have relative short persistence in the environment (Barr & Needham 2002), have been detected in surface water bodies (Larson et al. 1998, Gárcia de Llasera & Bernal-González 2001, Cerejeira et al. 2003, Guest et al. 2006, Wilson & Foos 2006); furthermore, these detection records frequently exceed established reference safety levels (e.g. EC 1998). The insecticide Methomyl [S-methyl N-(methylcarbamoyloxy) thioacetimidate] and the herbicide Propanil (3,4-dichloropropioanilide) are examples of such agrochemicals.

Methomyl is a monomethyl carbamate widely used to control a large range of insects and spider mites through direct contact and ingestion (Tomlin 2001). Carbamates reversibly inhibit cholinesterase enzymes, such as acetylcholinesterase (AChE), which hydrolyses the cationic neurotransmitter acetylcholine at very high rates; these pesticides inactivate the enzyme through carbamoylation of its active serine, hence compromising the normal neurotransmission function (Quinn 1987). Inhibition of AChE has been used as a specific biomarker for exposure to carbamates in *Daphnia*, however, these are able to significantly inhibit other esterases (Barata et al. 2004), and the relationship between the biomarker and the observed toxic effect at the individual level was already shown to be dependent on the acting chemical (Printes & Callaghan 2004). Cues though

exist over the toxicity of carbamates to *Daphnia*, but further information is needed that provides valuable insights on the mechanisms, e.g. on other target sites for these chemicals and on the actual pathways linking AChE inhibition and related life-history parameters onserved at the individual level, such as death of the animal.

Propanil is a highly selective anilide that is commonly applied in the post-emergency of rice and acts through direct surface contact to control grass- and broadleaf weeds (Tomlin 2001). Its specific mechanism of toxicity in target species involves an enzyme-mediated process of disruption of the electron flow in the Photosystem II, therefore inhibiting the light reaction of photosynthesis (e.g., Mitsou et al. 2006). The ability of low doses of Propanil to induce deleterious effects in *Daphnia* survival, life-history, egg hatchability impairment and feeding mechanisms was already reported (e.g., Villarroel et al. 2003, Pereira et al. 2007). Information on cellular and sub-cellular toxicological pathways of the chemical in non-target systems is scattered but a few focused studies are available in the literature: e.g., Cuff et al. (1996) showed immunotoxic effects induced by Propanil on mice thymus; Propanil and its major metabolite 3,4-Dichloroaniline (DCA) showed myelotoxicity in mice and human cord blood progenitor cells (Blyler et al. 1994, Malerba et al. 2002); DCA was shown to induce methemoglobinemia in rats (Guilhermino et al. 1998), as well as oxidative stress, disruption of antoxidant and GST enzymes, and lipid peroxidation in fish liver (Li et al. 2003).

Typically, the study of the potential impact of xenobiotics in non-target organisms addresses whole-organism or population responses, either attempting for exposure (e.g. biomarkers) or effects (e.g. perturbations in fitness) assessment (Neumann & Galvez 2002). Despite providing a valuable insight and useful information e.g. for regulatory purposes, such an assessment can rarely be accurately discriminative of the toxicity mechanisms underlying the observed response. The integration of genomic-based tools in Ecotoxicology, i.e. Ecotoxicogenomics, is becoming a promising approach that can provide a broad view over how living systems respond to a given toxicant stimulus and relevant information for the ecological risk assessment of chemicals (Neumann & Galvez 2002, Snape et al. 2004, Robbens et al. 2007). Transcription profiling using microarrays (first described by Shena et al. 1995) is one of the most prominent genome-wide technologies within Ecotoxicogenomics since it allows e.g. a comprehensive overview on the changes in gene expression of a given organism when exposure to a given stressor occurs. With such an approach, one can gather relevant information at least on the relationship between exposure and effects, on the sub-cellular molecular mechanisms of toxicity and for the identification of accurate biomarkers of

exposure (Neumann & Galvez 2002). Very recently, DNA microarrays-related techniques have been successfully used to address gene expression responses of *Daphnia magna* to different environmental toxicants, including pharmaceuticals, heavy-metals, pesticides and PAH's (Heckmann et al. 2006, Soetaert et al. 2006, Soetaert et al. 2007, Watanabe et al. 2007, Connon et al. 2008).

With the present study, we expected to get an insight on the molecular mechanisms underlying the toxicity of the insecticide Methomyl and the herbicide Propanil on *Daphnia magna*, and to discriminate whether these mechanisms could be chemical-specific rather than reflect general responses to toxicant-induced stress. The toxicant effects of both chemicals at the individual-level were assessed using a standard immobilisation assay. Considering the obtained dose-response curve, acute exposures of *D. magna* newborns to single doses of the pesticides were conducted, so that the exposure-related differential gene expression could be addressed; for this purpose, untreated and toxicant-treated mRNA transcripts were hybridised onto a customised *D. magna* cDNA microarray. By using such a toxicant-specific gene expression profiling approach, a holistic view on the molecular effects driven by each pesticide becomes available, promisingly providing relevant information on the occurring toxicity mechanisms.

MATERIAL & METHODS

Daphnia magna were obtained from the Water Research Centre (WRc), Medmenham, UK, and were cultured at the University of Reading, UK for at least 2 years before testing. Cultures were initiated with third brood offspring from a single female. Groups of 15 *Daphnia* were maintained in 1.2 L of reconstituted water, which was prepared by dissolving 195.87mg $CaCl_2.2H_2O$, 82.20mg $MgSO_4.7H_2O$, 64.80mg $NaHCO_3$, 5.80mg KCl and 0.002mg Na_2SeO_3 in 1 L of reverse osmosis water (e.g. ISO 1996). Initial hardness (measured as $CaCO_3$), pH, conductivity and dissolved O_2 ranged from 140 to 160 mg/L, 7.9 to 8.2, 370 to 450µS/cm and 7.9-8.2 mg/L, respectively. A $16h^L$: $8h^D$ photoperiod was used and the room temperature was kept at 20±1ºC. The culturing medium was renewed at least weekly, when a standard organic extract, Marinure (Glenside Organics Ltd, Throsk, UK) was added at a concentration of 0.2 mL/L (Baird et al.1989). Cultures were fed on a daily basis with suspensions of dried bakers yeast at a final rate of 0.04mg/L (Westmill Foods Ltd, Maidenhead, UK) and of the unicellular green algae *Chlorella vulgaris* var *viridis*, equivalent to 0.5–2.0 mgC^{-1} depending on the age of the *Daphnia*. Third to fifth brood neonates were used for testing.

Chemicals and range-finding assays

Methomyl (Pestanal®, 99.5% purity) and Propanil (Pestanal®, 99.7% purity) were supplied by Sigma Aldrich (Seelze, Germany). Stock solutions were freshly prepared prior to experiments by directly dissolving Methomyl or Propanil in culture medium. The acute toxicity of each pesticide to *D. magna* was assessed following the related OECD (2004) standard protocol. The 48-h exposures were carried out under a static design using twenty neonates (<24h-old) per treatment. Five animals were randomly assigned into four replicates per treatment. Incubation conditions were as described for culturing. The tests were conducted in glass beakers, each containing 50ml culture media (negative control)/pesticide test solution. Dissolved oxygen and pH were monitored at the beginning and the end of the tests for validation purposes. Immobilised individuals were counted at 48-h exposure. Effect-concentrations, EC_1 to EC_{50} (Table I), were estimated via Probit analysis (Finney 1971).

Information on the microarray

A customised cDNA microarray with over 15000 gene fragments representing more than 5000 unique Expressed Sequence Tags (EST's) was used in this study: (i) ca. 10000 clones randomly selected from a cDNA library of unexposed *D. magna* (Daphniabase, http://daphnia.nibb.ac.jp); ca. 1500 clones derived from suppressed subtractive hybridisation (SSH) between adults and juveniles (Soetaert et al. 2006); and ca. 3500 clones derived from SSH on populations exposed to selected stressors such as cadmium, lufenuron, pH, hardness, kerosene and ibuprofen (Heckmann et al. 2006, Connon et al. 2008).

Microarray experiment

Neonate *Daphnia magna* (< 24-h old), differing less than 3-h in age in order to reduce exposure variation, were obtained from the bulk cultures (see above) and were exposed for 48 hours to the chemicals (1-L test solution). A randomised block design with three treatments was followed: negative control, Methomyl EC_1 and Propanil EC_1; see TABLE V.I for the related nominal concentrations. Five replicates were used per block and thirty neonates were randomly assigned to each replicate. After the 48-h static exposure, the organisms were harvested into micro-centrifuge tubes with 150µL RNA*later*® (Ambion, UK) for immediate preventing RNase activation (following Heckmann et al., 2007) and then stored at -80ºC until further processing.

Total RNA was extracted using the RNeasy Mini kit with on-column DNase treatment (Qiagen, UK) to remove any traces of genomic DNA following the manufacturer's instructions. RNA

concentrations were determined by spectrophotometry using GeneQuant Pro (Biochrom, UK), while RNA integrity was verified on a BioAnalyzer 2100 (Agilent Technologies, UK). Total RNA (400ng) from each sample was then amplified using the *Aminoallyl Message Amp aRNA Amplification Kit* (Ambion, UK). Reference material was created from the amplified samples. 10µg aRNA taken from each sample was mixed and divided into 16 aliquots. Aliquots were dye-labeled and purified using *Aminoallyl Message Amp aRNA Amplification Kit* (Ambion – UK) purification module. Alexa Fluor dyes, 555 and 647 respectively, were used in place of cy3 and cy5 supplied with the kit. Following purification, aliquots were mixed and the large pool was quantified. 5µg aliquots of reference material were divided into 16, 0.5ml microfuge tubes and stored at -20°C for subsequent hybridization. Treated samples (10µg) were prepared using the same kit, and Alexa Fluor dyes were used in place of those supplied. Purified samples were quantified as above, and 5µg aRNA was mixed with each 5µg reference sample prior to hybridisation. Slides were pre-hybridised in a solution containing 50% v/v deionised formamide, 5x sodium chloride-sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) and 1% w/v bovine serum albumin (BSA) (Sigma-Aldrich, UK), and incubated at 42°C in a Techne HB-1 Hybridiser (Techne Ltd., UK) for 1 h. A 45 µl hybridisation probe solution was prepared with 22.5 µl deionised formamide, 5x SSC, the labelled aRNA mix (combined sample and reference pool cDNA) and a hybridisation block mix containing 0.1% SDS, 0.5 mg ml-1 polyA RNA (Sigma-Aldrich, UK), 0.5 mgmL^{-1} yeast tRNA, 0.5 mg mL^{-1} salmon sperm DNA, 25 µgmL^{-1} human and 25 µgmL^{-1} mouse Cot-1 DNA (Invitrogen, UK). The probes were hybridised to each microarray, in batches of three slides, corresponding to controls and respective treatments, under a 2560 lifterslipTM (Implen, UK). The slides were then placed in an airtight plastic box and incubated at 42°C in a Techne HB-1 Hybridiser (Techne Ltd., UK) for 16 h. After hybridisation, the slides were washed in a series of buffer solutions (2xSSC; SSC and 0.1%SDS; 0.1xSSC; 0.05xSSC) and 100% Isopropanol.

Bioinformatics

Readily after the hybridisation process was completed, the slides were scanned on GenePix Professional 4200A scanner and analysed using GenePixPro v.6 software (Axon Instruments/Molecular Devices, UK). During the scans, Auto-PMT function was used (saturation tolerance 0.005%, as recommended by manufacturer) to avoid excess of saturated pixels. Spot intensities were local background adjusted (median values), and those spots with signal-to-noise ratio less than three or with more than 50% of saturated pixels were removed from further analysis as unreliable. Intensity data over each scanned microarray were imported into latest version TM4

software for pre-processing and analysis (Saeed et al., 2003). Spot intensities were adjusted through log_2 transformation and normalized using block lowess (Yang et al. 2002). Statistically significant differences (One-Way ANOVA and t-test; $P \leq 0.05$) and more than 2-fold change, between gene expression in control and pesticide-treated conditions, were used as combined criteria on the identification of differentially expressed genes. Expression levels of all genes meeting these criteria were considered for further analysis as the median within replicates of the intensity ratios (Toxicant treatment vs untreated Control). Sequences were annotated following BLASTX homology search against GenBank (http://www.ncbi.nlm.nih.gov/), and were only considered if a BLAST hit meeting an expect value (E-value) $<10^{-5}$ and a score >50 could be registered. The detailed records of successful annotations meeting these feasibility criteria - comprising all clone ID's and respective NCBI accession numbers and species' match - can be found in APPENDIX V.I (Methomyl-related gene expression) and APPENDIX V.II (Propanil-related gene expression). Functional information was gathered over this filtered list of annotated sequences using the Protein knowledgebase Uniprot freely available through the web site http://beta.uniprot.org/.

RESULTS

The immobilisation results obtained after the 48-h exposure to Methomyl and Propanil provided a robust range of records to estimate ECs following the Probit method (TABLE V.I). This procedure was useful on the determination of most appropriate nominal chemical concentrations for use in the actual microarray experiment by providing a direct link between toxicity at the individual level and gene expression.

TABLE V.I: Estimated Effect Concentrations (ECs) regarding the immobilisation of *Daphnia magna* after a 48-h exposure to Methomyl and Propanil.

	[Propanil] (95% Confidence Interval) µg/L	**[Methomyl] (95% Confidence Interval) µg/L**
EC_1	3635.87 (3023.81 - 4009.76)	10.5237 (8.82195 - 11.6998)
EC_2	3785.70 (3208.38 - 4138.85)	11.0730 (9.45002 - 12.1969)
EC_3	3883.96 (3330.68 - 4223.66)	11.4363 (9.86919 - 12.5260)
EC_4	3959.54 (3425.35 - 4289.10)	11.7175 (10.1952 - 12.7814)
EC_5	4022.11 (3504.04 - 4343.45)	11.9512 (10.4672 - 12.9944)
EC_{10}	4244.52 (3785.21 - 4538.85)	12.7897 (11.4460 - 13.7664)
EC_{20}	4530.36 (4145.62 - 4799.33)	13.8842 (12.7153 - 14.8084)
EC_{40}	4942.90 (4642.18 - 5215.23)	15.4956 (14.4973 - 16.4815)
EC_{50}	5131.94 (4850.42 - 5431.50)	16.2459 (15.2658 - 17.3427)

The estimated EC1s were shown to be sufficient to generate changes in gene expression of *D. magna*. From the 15000 cDNA clones (~5000 unique ESTs) spotted in the microarray, 768 were found to be differentially expressed by the exposure to Methomyl and/or Propanil. Propanil was more effective in promoting gene expression changes than was Methomyl, i.e., Propanil exposure resulted in the significant change of 738 genes whereas Methomyl exposure resulted in the significant change of 624 genes. More genes were up-regulated by Propanil while Methomyl promoted a comparatively stronger down-regulation of genes (FIGURE V.1). The full data matrix is provided in APPENDIX V.I.

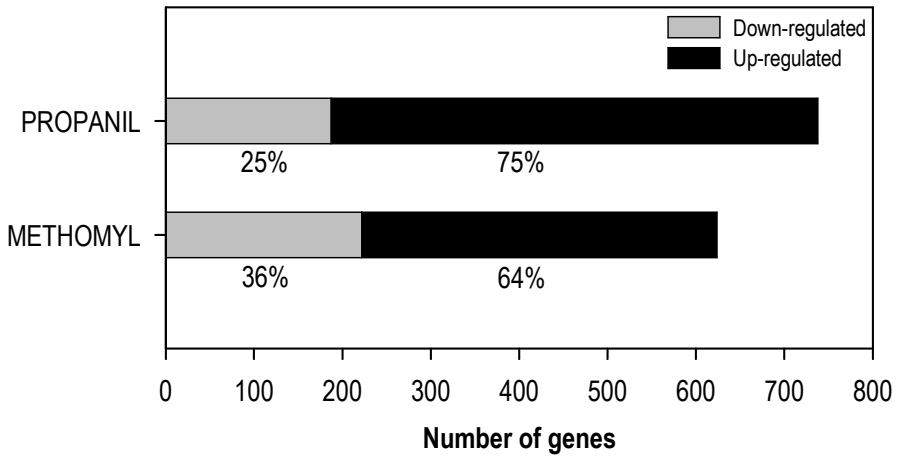


FIGURE V.1 | Total number of differentially expressed genes (ESTs), regarding *D. magna* exposures to Methomyl and Propanil. The bars provide a comparative view on up- (black) and down-regulated (grey) genes within each chemical exposure. The relative proportions between up- and down-regulated genes within each chemical are depicted below the bars.

A global comparison of the two datasets (Methomyl and Propanil) indicates that most of the differentially expressed genes respond similarly to both chemicals i.e. a large component of the gene expression response to either chemical may be related to general mechanisms of cellular response to chemical exposure (FIGURE V.2). Some chemical-specific patterns can also be depicted using this graphical approach: (i) very few genes were differentially expressed following a chemical-specific down-regulation pattern; (ii) there was a considerably large group of genes which were up-regulated by one or another pesticide, suggesting that mechanisms of chemical-specific cellular responses to stress may have been found.

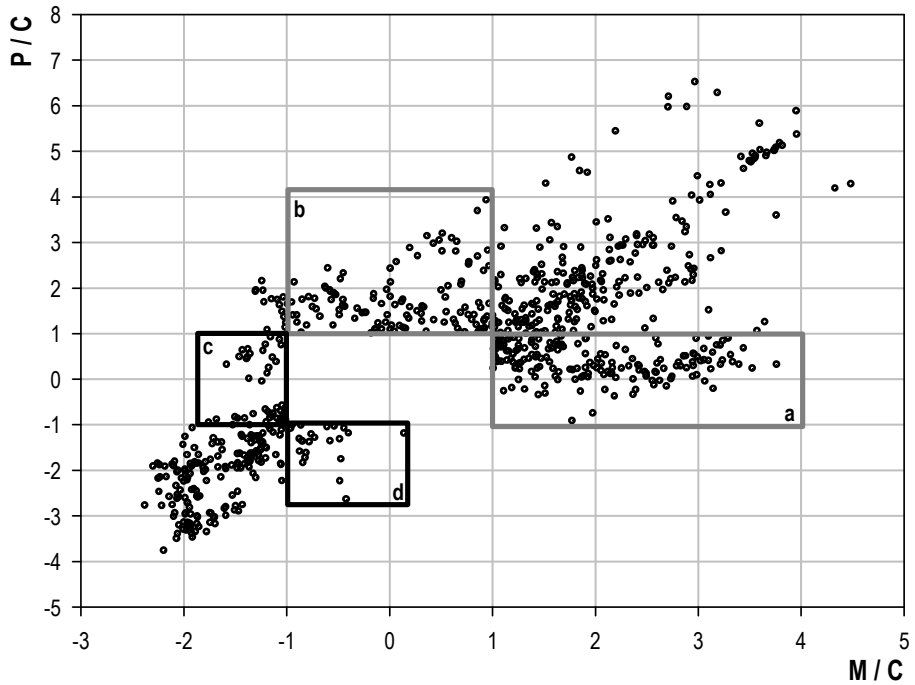
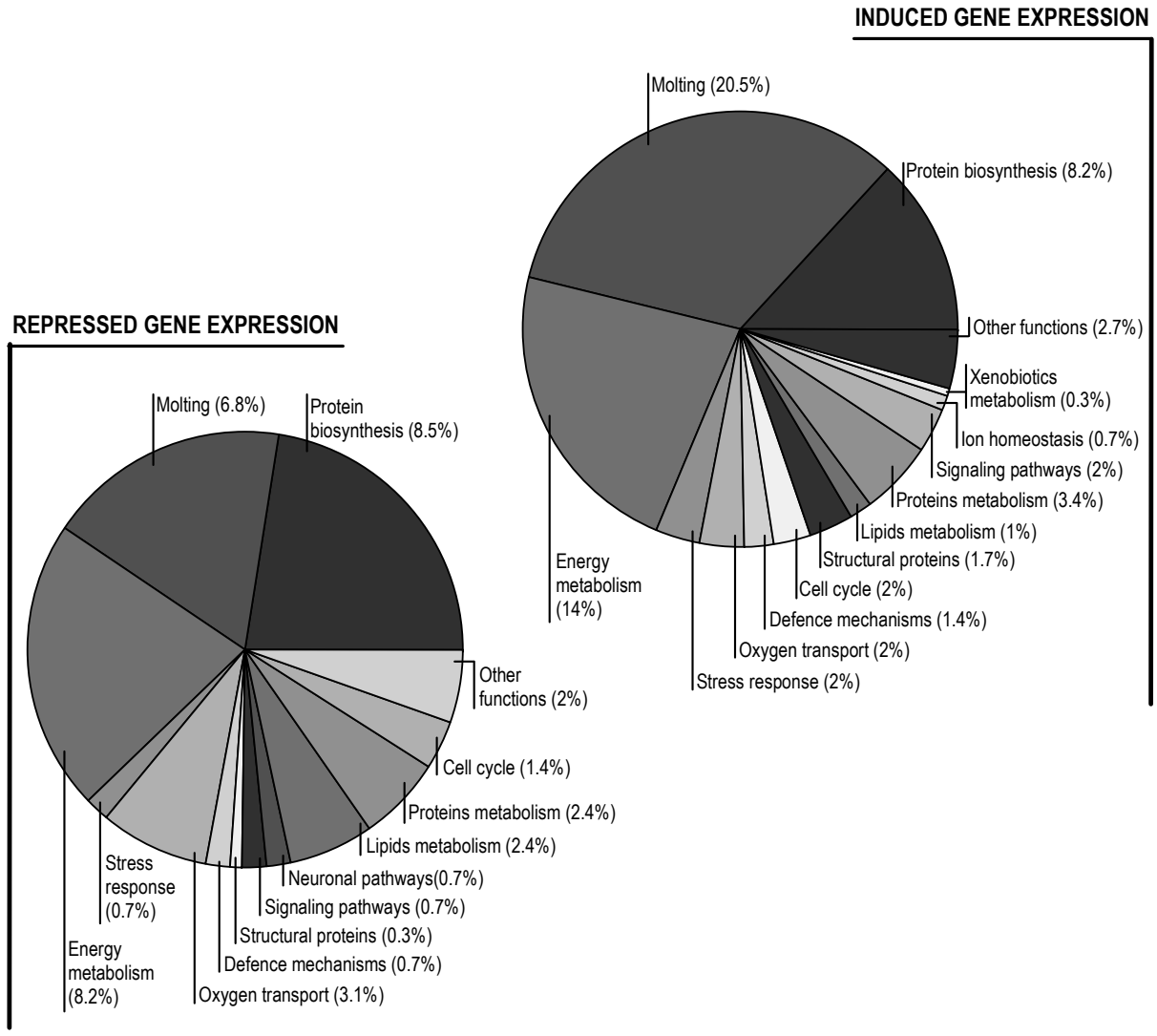


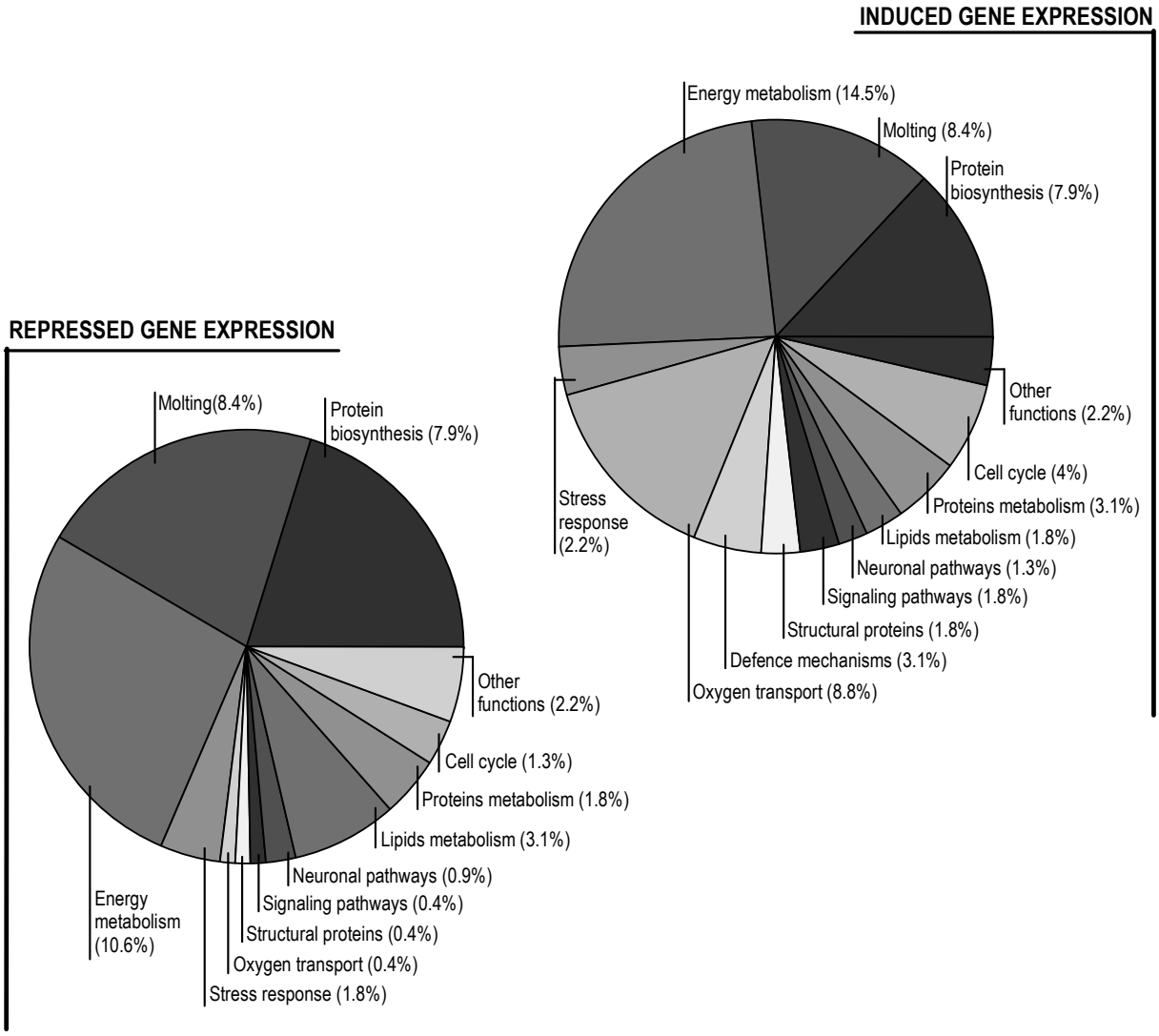
FIGURE V.2 | Relative gene expression of *D. magna* exposed to Methomyl and Propanil. Each dot refers to a gene where a 2-fold change in expression was observed after at least one of the chemical exposures vs. control. M/C and P/C stand for the log_2 transformed signal intensity ratios between treated samples (M and P) and untreated control (C). Grey squares indicate genes which were up-regulated exclusively by Methomyl (a) or by Propanil (b), and black squares depict chemical-specific down-regulation (respectively, c and d).

Out of the 768 sequences differentially expressed by *D. magna* after exposure to Methomyl and/or Propanil, only 354 were successfully annotated (APPENDIX V.I and V.II). These annotated genes were then analysed for their functional roles and grouped in accordance so that the gene expression profile of each pesticide and the overall chemical-dependent molecular effects could be addressed (FIGURE V.3,4). The Methomyl exposure up-regulated 182 genes, whereas only 111 spots were shown as down-regulated genes (FIGURE V.3). Molting, protein metabolism and energy metabolism were clearly the biological processes assigning more differentially expressed genes. Genes associated with molting represented 20.5% of the genes differentially expressed following Methomyl exposure. Genes involved in protein biosynthesis were up- and down-regulated by the chemical in identical proportions (around 8%), whereas the up-regulation of genes related with energy metabolism was more relevant (14%) than the down-regulation of the related genes (8.2%). Genes coding for proteins related with oxygen transport activities and lipids metabolism were more repressed than induced by Methomyl; and the contrary could be observed for structural proteins and defence mechanisms. Exclusive down-regulation was registered for genes specifically associated with neuronal pathways, as well as exclusive up-regulation of genes involved in xenobiotics metabolism and ion homeostasis should additionally be noticed.



Protein biosynthesis: Ribossomal proteins; proteins folding and transport to and within ER and Golgi; transcription/ translation factors | **Moulting:** cuticular proteins; chitin-binding proteins and precursors; constituents of the peritrophic membrane | **Energy metabolism:** mitochondrial genome; enzymes related with the glycolysis and the respiratory chain | **Stress response:** HSPs; Ferritin; Superoxide dismutase | **Oxygen transport:** Haemoglobin | **Defence mechanisms:** Lectin; Preproadipsin | **Structural proteins:** Actin; Thymosin; muscular proteins; Collagen | **Lipids metabolism:** Proteins related with lipid transport; phospholipase activators; Vitellogenin | **Proteins metabolism:** Carboxypeptidases; Trypsin; Chymotrypsin; Proteinases; Protein phosphatises | **Xenobiotics metabolism:** Sulfotransferase | **Signaling pathways:** Kinases; Rhodopsin; chemosensory proteins | **Neuronal pathways:** Carboxylesterases; Doughnut | **Ion homeostasis:** Ion exchangers across the plasma membrane | **Cell cycle:** Proteins with active role in cell division, actin filament organisation, DNA replication, and cell shape regulation | **Other functions:** Carbonic anhydrase; phosphopantothenoylcysteine synthetase; Selenoprotein; Angiomotin; translationally-controled tumor protein-like protein; TRE genomic sequence; thioesterases; oxireductases.

FIGURE V.3 | Proportional view of inducing (up-regulated genes) and repressing (down-regulated genes) gene expression patterns regarding the *Daphnia magna* exposure to Methomyl. Percentages in brackets refer quantitatively to the representativeness of the function within the total number of differentially expressed genes after the chemical exposure. The text box below the figure provides information on most of the proteins which were assigned to each functional mechanism/pathway.



Protein biosynthesis: Ribossomal proteins; proteins folding and transport to and within ER and Golgi; transcription/ translation factors | **Moulting:** cuticular proteins; chitin-binding proteins and precursors; constituents of the peritrophic membrane | **Energy metabolism:** mitochondrial genome; enzymes related with the glycolysis and the respiratory chain | **Stress response:** Ferritin; Superoxide dismutase; Peroxinectin | **Oxygen transport:** Haemoglobin | **Defence mechanisms:** Lectin; Preproadipsin; Cystatin | **Structural proteins:** Thymosin; Myosin; Innexin; Collagen | **Lipids metabolism:** Proteins related with lipid transport and biosynthesis; Vitellogenin | **Proteins metabolism:** Carboxypeptidases; Trypsin; Chymotrypsin; proteinases| **Signaling pathways:** Kinases; Rhodopsin; developmental proteins | **Neuronal pathways:** Carboxylsterases; Syntaxin; Dopa decarboxylase; Doughnut | **Cell cycle:** Proteins with active role in cell division, cell differentiation, actin filament organisation, DNA replication, and cell shape regulation | **Other functions:** Carbonic anhydrase; phosphopantothenoylcysteine synthetase; glutamine synthetase; Selenoprotein; oxireductases; Annexin; Thioesterases.

FIGURE V.4 | Proportional view of inducing (up-regulated genes) and repressing (down-regulated genes) gene expression patterns regarding the *Daphnia magna* exposure to Propanil. Percentages in brackets refer quantitatively to the representativeness of the function within the total number of differentially expressed genes after the chemical exposure. The text box below the figure provides information on most of the proteins which were assigned to each functional mechanism/pathway.

The exposure to the herbicide Propanil promoted the differential expression of 293 sequences i.e. 182 were significantly up-regulated while 111 where significantly down-regulated. This pesticide was shown to affect gene expression primarily within functional mechanisms related with molting, protein biosynthesis, energy metabolism and oxygen transport (FIGURE V.4). Genes involved in the energy metabolism were highly up- and down-regulated (14.5% and 10.6% of all differentially expressed genes, respectively). Propanil was able to promote similar induction and repression of genes associated to the processes of molting and protein biosynthesis. High induction of oxygen transport by Propanil was noticed (8.8% up-regulated out of all differentially expressed genes) regardless the small percentage of related genes (0.4%) which were down-regulated. If one excludes lipids metabolism, a general trend for up-regulation of genes which were assigned to the remaining functional processes was depicted considering that the related down-regulated genes had consistently low representation within all differentially expressed. No genes belonging to defence mechanisms were repressed; on the other hand, up-regulation of genes which were assigned to this biological function was considerable (3.1% of all differentially expressed genes). Unlikely Methomyl, Propanil was not able to promote the differential expression of genes related with either ion homeostasis or xenobiotics metabolism.

Few genes were toxicant specific (Table II) and more genes were up-regulated than down-regulated within these (only 4 and 3 genes were specifically down-regulated by Methomyl or Propanil, respectively). Methomyl-specific expression includes genes coding for generalised biological processes such as signalling pathways, ion homeostasis and proteins metabolism. A single gene coding to the protein sufotransferase was found that seems to be directly related with the exposure to the xenobiotic Methomyl (up-regulated). Propanil was able to induce the chemical-specific expression of genes coding for proteins within generalised biological processes such as neuronal and signalling pathways, cell cycle, protein biosynthesis and lipids metabolism. A cystatin precursor, which is involved in cell defence mechanisms, was specifically up-regulated by Propanil whereas the expression of the stress-related protein Peroxinectin was down-regulated by the chemical. A few differentially genes belonging to none of the established biological mechanisms/processes, and coding for proteins with different functions, could also be specifically linked to the exposure of *Daphnia magna* to either pesticides.

TABLE V.II: Summary of specific gene expression strictly related to the exposures of *Daphnia magna* to Methomyl or Propanil. The genes depicted were found to be exclusively up- or down-regulated by one or another pesticide; those genes which met this criterion but were up- and down-regulated within each chemical were excluded from this summary. The mechanisms/processes to which each gene can be related were specified in square brackets.

Methomyl		**Propanil**	
Up-regulated genes			
Actin	[Structural proteins]	Acyl-CoA desaturase	[Lipids metabolism]
Angiomotin	[Other functions]	Cystatin precursor	[Defence mechanisms]
cAMP-regulated protein	[Signalling pathways]	Dopa decarboxylase	[Neuronal pathways]
Chemosensory protein	[Signalling pathways]	Glutamine synthetase	[Other functions]
Chloride/bicarbonate AE	[Ion homeostasis]	Innexin 2	[Structural proteins]
Ghitm-prov protein	[Other functions]	Katanin 60	[Cell cycle]
NA,K-ATPase	[Ion homeostasis]	Myosin light chain	[Structural proteins]
Pros45 proteosome subunit	[Proteins metabolism]	NFkB protein	[Protein biosynthesis]
Putative muscular protein20	[Structural proteins]	Ornithine decarboxylase	[Cell cycle]
Serine collagenase 1[p]	[Proteins metabolism]	Syntaxin 6	[Neuronal pathways]
Sulfotransferase	[Xenobiotics metabolism]	Vang-like protein 2	[Signalling pathways]
Tc tumor protein	[Other functions]		
Down-regulated genes			
Epididymal secretory protein[p]	[Other functions]	Headcase protein	[Other functions]
Protein phosphatase 1K	[Proteins metabolism]	Peroxinectin	[Stress response]
PHB depolymerase	[Other functions]	Trehalose transporter	[Other functions]
Serine-type protease inhibitor	[Proteins metabolism]		

[p] Percursor

DISCUSSION

In this study, we linked chemical-induced immobilisation rates with gene expression analysis by exposing *Daphnia magna* neonates to concentrations which were previously estimated to immobilise 1% of the experimental organisms (EC_1). These estimated concentrations are far below the levels that are likely to cause lethal toxicity, however and for both pesticides, these concentrations are likely to cause hazardous effects under a chronic exposure [see LOECs obtained for reproduction and growth parameters obtained by Pereira et al. (2007) and Pereira & Gonçalves (2007)]. When comparing our data on the chemical-induced immobilisation rates (e.g. EC_{50}) with the literature, some inconsistency in species sensitivity could be depicted. Considering that EC_{50}s higher than 24µg/L were previously reported for Methomyl (Tomlin 2001, Pereira & Gonçalves 2007), the particular *D. magna* clonal lineage used in this study showed a relative enhanced sensitivity to the chemical (EC_{50} = 16.2µg/L). The estimated Propanil EC_{50} (5.1µg/L) was consistent with that obtained by Villarroel et al. (2003), but higher than the EC_{50} of 3.5µg/L reported by Pereira et al. (2007).

Regardless the factors that may contribute to these differences in toxicity (e.g. genotype sensitivity, culturing conditions, purity of the toxicant solution), the actual testing of effects might provide more accurate reference concentrations for further use in microarray experiments. The gene expression output obtained here (more that 600 gene sequences were differentially expressed in either chemical exposure) indicate a fairly appropriate defining of exposure concentrations.

Arthropods growth occurs through a process of periodic shedding of the exoskeleton synchronised with the regeneration of the cuticle (Ruppert & Barnes 1996). Gene sequences related to the molting process, such as those generally involved in new exoskeleton synthesis (e.g. various cuticle proteins, chitin-binding proteins, structural constituents of cuticle) or in old exoskeleton breakdown (e.g. chitin deacetylase, and eventually carboxipeptidases and serine proteases), are likely to interact for the success of exuviation, and were highly differentially expressed after the exposure of *D. magna* to both Methomyl and Propanil. Molting in *Daphnia* is regulated by a multi-hormonal system, where the immediate controllers are ecdysteroids (Chang et al., 1993). Some xenobiotics, including pesticides, were already proven to affect molting in *Daphnia* possibly through interference with this endocrine pathway (e.g. Zou & Fingerman 1997). Genes related with these steroid hormones were not found differentially expressed after the exposures, which indicates that the pesticides were not able to disrupt the hormonal regulation of molting. Notwithstanding, the expression patterns of molting-related genes seems to point towards some changes in the process due to exposure to either pesticides. Methomyl strongly up-regulated molting-related genes, including various structural constituents of cuticle, cuticular proteins, and chitin deacetylases, suggesting that the molting cycle was somewhat accelerated in response to the chemical exposure. Conversely, Propanil tended to promote equivalent induction and repression of molting-related genes; assuming that down-regulation of these genes means lower synthesis of cuticle components i.e. a chemical-induced delay in the molting cycle, it could be that daphnids undertake a compensatory-like strategy by enhancing the cuticle components synthesis, and hence the complementarily observed up-regulation of molting-related genes.

Ribosomes support growth in the cell since they are key actors in protein biosynthesis (Stryer 1999). Considering that RNA makes up 50-60% of the ribosome, which has a steady-state level comprising 80-90% of the total cellular RNA, and that *Daphnia* are fast-growing crustaceans with high relative RNA content (ca. 10%RNA per unit of weight) (Elser et al. 2000), the relevance of protein biosynthesis within our dataset in terms of differentially expressed genes should not be

surprising. In fact, this biological process assigned more than 15% of all differentially expressed genes after *D. magna* exposure to either Methomyl or Propanil, and the large majority of these genes code for ribosomal proteins. Both pesticides promoted similar levels of up- and down- regulation of protein biosynthesis-related genes (ca. 8%). Daphnids seem to be able to compensate chemical-induced gene repression with an over-expression that might allow them continue growing. In this way, both pesticides also induced the expression of genes coding for structural proteins, which will generally support cell and tissue growth.

Along with protein synthesis, growth also requires energy (ATP). Survival and growth in young daphnids depends on their energy budget, and environmental toxicants, such as pesticides, are known to reduce cellular energy budgets (DeCoen and Janssen 2003). Either Methomyl or Propanil promoted differential expression of energy-related genes. Induction of ATP synthase and enzymes involved in glycolysis and in the respiratory chain suggests enhanced needs of energy directly in response to the toxicants stress or eventually compensating for the observed down-regulation of energy related genes. In addition, the digestive enzyme α-amilase and diverse lipoproteins were up-regulated after exposure of *D. magna* to both pesticides; while the induction of the former enzyme may follow the need for carbohydrate breakdown for further energy production, induction of lipid-related gene expression is likely to indicate lipid reserves mobilisation to maintain homeostasis during the toxicant exposure (DeCoen & Janssen 2003). Vitellogenin, a four-subunit lipoprotein which is the precursor of the major yolk protein vitellin (Kato et al. 2004, Tokishita et al 2006), as well as vitellogenin fused with Cu/Zn superoxide dismutase (the superoxide dismutase should have a role in intermediate detoxification of superoxides resulting from vitellogenin metabolism - Kato et al. 2004), were also consistently induced after the toxicant exposures. Previous work using mature *D. magna* females, eggs or embryos, has been carried that found differentially expression in genes coding for both proteins in response to environmental toxicants (Soetaert et al. 2006, Tokishita et al. 2006, Soetaert et al 2007). These authors relate the gene expression changes with chemical-induced impairment of reproduction, e.g. in egg and embryo development, and highlight this gene as good candidate toxicity biomarker; however, we used neonate daphnids in the present study and hence such a relationship seems unlikely. One may speculate that vitellogenin can function as a general liprotein involved in lipid reserves mobilisation as a contribution for energy production, but further experimental evidence is needed to clarify vitellogenin function in *Daphnia* and hence eventually validate its use as a biomarker of toxicant-induced reproduction impairment.

Despite the recognised toxicity of its superoxide anions, oxygen fuels metabolic needs for survival and growth; the biological process of oxygen transport is hence crucial in the generation of energy for sustaining cell metabolism (Stryer 1999). In *Daphnia*, oxygen transport is ensured by extracellular, multi-subunit assembled Haemoglobin (Hb), which is encoded by four well-characterised Hb genes (Kimura et al. 1999, Nunes et al. 2005). Daphnids are hypoxia-tolerant since they are able to strongly increase Hb synthesis and oxygen affinity of Hb in response to low environmental oxygen levels (Kobayashi et al. 1990, Seidl et al. 2005). Both Methomyl and Propanil induced the expression of the four Hb *D. magna* genes, which indicates similarity between juvenile and adult Haemoglobin, at least at the transcriptional level [see Terwilliger & Ryan (2001) for discussion on a possible ontogenetic shift in the protein expression according to the developmental stage of the organism]. Hb synthesis seems particularly critical after exposure to Propanil (9% of the differentially expressed sequences were up-regulated Hb sequences), suggesting that the pesticide is able to impair oxygen transport in *D. magna*. Rider & LeBlanc (2006) found that the triazine herbicide Atrazine induced Hb expression and/or increasing concentration in *Daphnia* although, if initially hormonal pathways were hypothesised to be involved in this effect, the experimental evidences were not confirmative. Hb adducts were already identified in rats exposed to anilides (Beyerbach & Sabbione 1999) and in agricultural workers exposed to Propanil (Pastorelli et al. 1998); the increase of methemoglobin levels in rat blood cells was also observed after exposure to Propanil (McMillan et al. 1990). Albeit the molecular mechanisms behind the impairment of oxygen transport by pesticides could not yet be fully clarified, the Hb genes seem promising candidates for use as a biomarker of general toxicant exposure in *Daphnia*.

Genes related to defence mechanisms and stress response tended to be essentially up-regulated by both pesticides although this pattern was of higher meaning after exposure to Propanil. These pathways involve the expression of genes coding for galactose-binding C-type lectins (cell agglutination/adhesion), cystatins (protease inhibitors), and ferritins. Invertebrates are thought to lack adaptive immune systems and rather have innate immunity defence mechanisms against unspecific antigens (Muta & Iwanaga 1996, Janeway & Medzitov 2002, Little et al. 2003); the general immune response may involve hemolymph coagulation driven by specialised hemocytes, where lectins and cystatins cooperate (Muta & Iwanaga 1996). Whether the immune response depicted in this particular microarray experiment can be directly related with the chemical exposure to the pesticides remains unclear and further studies need to be carried in order to better understand how pesticides can affect *Daphnia* immunity. Ferritins are generally involved in the storage and scavenging of iron

although they were shown transcriptionally up-regulated after exposure of *Daphnia magna* to other metals (Poynton et al. 2007). These proteins have also a role in assisting oxidative stress processes (Hintze & Theil 2005), therefore the induction of ferritin by Methomyl and Propanil should be an indirect effect mediated by oxidative stress, rather than a direct response to the exposure. In fact, the respiratory proteins were also up-regulated by both pesticides in this study, indicating a need for energy production (see above); the superoxide anion is highly produced in the respiratory chain (Stryer 1999) thus an additional activity of the oxidative stress-response system would be expectable. Moreover, experimental evidence exists of the ability of either Methomyl or Propanil to induce oxidative stress (El-Khawaga 2005, Milatovic et al. 2006, Moraes et al. 2007).

The up-regulation of ion homeostasis (Chloride/bicarbonate anion exchanger; Na,K-ATPase) and sulfotransferase, a protein strictly related with the metabolism of xenobiotic chemicals, was a unique signature of Methomyl exposure. The inhibition of active sodium uptake in *D. magna* through blocking of Na,K ATPase by silver was already reported in literature (Bianchini & Wood 2003). One may speculate that Methomyl may interact in such a way with both the refereed ion regulators, and hence consider them candidate biomarkers of exposure to the pesticide, however further research is needed so that this hypothesis can be experimentally verified. Sulfotransferase assists the sulphate conjugation of several endogenous and xenobiotic compounds, including alcohols, thiols, and amines (Josephy 1997). By exposing *D. magna* to pyrene, Ikenaka et al (2006) have confirmed the tendency of aquatic invertebrates to biotransform xenobiotics in sulphate conjugates via sulfotransferase. This protein seems though to generally assist xenobiotics detoxification and its potential as a specific biomarker of exposure to carbamates would be unlikely. As a carbamate insecticide, Methomyl was expected to specifically promote damage across neuronal transmission. However it was the exposure to Propanil that elicited specific up-regulated gene expression within neuronal pathways: Dopa decarboxylase catalyses the conversion of dihydroxyphenylalanine (Dopa) to dopamine and 5-hydrotryptophan to serotonin in response to several endogenous or exogenous signals, and was already shown to be involved in insect cuticle maturation, neuronal regulation, pigmentation patterning and innate immunity (Hodgetts & O'Keefe 2006); Syntaxin 6 is a protein belonging to the synaptic vesicle release machinery and appears to regulate the presynaptic calcium channels activity (Wendler & Tooze 2001, Zamponi 2003). In fact, either Methomyl or Propanil repressed the expression of a carboxylesterase belonging to the AChE family (EF580101 – see APPENDIX V.I and V.II) hence confirming that both pesticides seem to impair neuronal pathways in *D. magna*. These data highlight the need for a deeper insight on the ability of pesticides belonging

to diverse chemical classes, and having diverse primary modes of action, to affect neurotransmission in aquatic non-target organisms such as *Daphnia*.

The analysis of gene expression using a DNA microarray approach can provide useful insights on the molecular/cellular mechanisms underlying the effects of stressful environmental conditions in the biota. The application of such an approach to *Daphnia magna*, a model organism in several Biology disciplines, including Ecotoxicology, may clarify the mechanisms of toxicity of xenobiotic chemicals which mediate the generally known toxicological effects at the individual-level. Moreover, as a genome-wide gene expression technology, DNA microarrays can be useful to identify accurate and sensitive biomarkers that enlarge the ecotoxicological toolbox available to predict toxicity, and are currently faced as promising techniques to address environmental chemicals risk assessment (Robbens et al. 2007). In this study, we addressed the effects of two widely used pesticides which toxicity to non-target organisms is not comprehensively characterised. Studies regarding the acute and chronic toxicity of Methomyl and Propanil to *D. magna* do exist (e.g. Villarroel et al. 2003, Pereira et al. 2007, Pereira & Gonçalves 2007) but information on the molecular/cellular mechanisms behind the effects noticed is rather very scarce. Indeed, unique *D. magna* gene expression profiles were found for either chemical, and potential candidates to biomarkers of exposure and/or effect were depicted by this study.

REFERENCES

Baird DJ, Soares AMVM, Girling A, Barber I, Bradley MC & Calow P (1989). The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. In: H Lokke, H Tyle & F Bro-Rasmussen (eds.), Proceedings of the First European Conference on Ecotoxicology. Lyngby, pp. 144-148.

Barata C, Solayan A & Porte C (2004). Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. *Aquatic Toxicology* 66: 125-139.

Barr DB & Needham LL (2002). Analytical methods for biological monitoring of exposure to pesticides: a review. *Journal of Chromatography B* 778: 5-29.

Beyerbach A & Sabionni G (1999). Biomonitoring of arylamines: haemoglobin adducts of aniline derivatives. *Biomarkers* 4: 229-236.

Bianchini A & Wood CM (2003). Mechanism of acute silver toxicity in *Daphnia magna*. *Environmental Toxicology and Chemistry* 22: 1361-1367.

Blyler G, Landreth KS, Lillis T, Schafer R, Theus SA, Gandy J & Barnett JB (1994). Selective myelotoxicity of Propanil. *Fundamental and Applied Toxicology* 22: 505-510.

Brown CD, Carter AD & Holis JM (1995). Soils and pesticide mobility. *In:* TR Roberts & PC Kearney (Eds.), *Environmental behaviour of agrochemicals* (Vol. 9 – Series: Progress in pesticide biochemistry and technology). John Wiley & Sons, West Sussex. pp 131-179.

Carter A (2000). How pesticides get into water – and proposed reduction measures. *Pesticide Outlook*: 149-156.

Cerejeira MJ, Viana P, Batista S, Pereira T, Silva E, Valério MJ, Silva A, Ferreira M & Silva-Fernandes AM (2003). Pesticides in Portuguese surface and ground waters. *Water Research* 37: 1055-1063.

Chang ES, Bruce MJ & Tamone SL (1993). Regulation of crustacean molting: a multihormonal system. *American Zoologist* 33: 324-329.

Connon R, Hooper H, Sibly RM, Lim F-L, Heckmann L-H, Moore DJ, Watanabe H, Soetaert A, Cook K, Maund SJ, Hutchinson TH, Moggs J, De Coen W, Iguchi T, Callaghan A (2008). Linking molecular and population stress responses in *Daphnia magna* exposed to cadmium. *Environmental Science and Technology* 42: 2181-2188.

Cuff CF, Zhao W, Nukui T, Schafer R & Barnett JB (1996). 3,4-Dichloropropionanilide-induced atrophy of the thymus: mechanisms of toxicity and recovery. *Fundamental and Applied Toxicology* 33: 83-90.

De Coen W & Janssen CR (2003). A multivariate biomarker-based model predicting population-level responses of *Daphnia magna*. *Environmental Toxicology and Chemistry* 22: 2195-2201.

EC (1998). Council Directive 98/83/EC on the quality of water intended for human consumption. Official Journal of the European Communities L330/32 – 05.12.1998.

El-Khawaga OAY (2005). Role of selenium on antioxidant capacity in methomyl-treated mice. *Journal of Physiology and Biochemistry* 61: 501-506.

Elser JJ, Sterner RW, Gorokhova E, Fagan WF, Markow TA, Cotner JB, Harrison JF, Hobbie SE, Odell GM & Weider LJ (2000). Biological stoichiometry from genes to ecosystem. *Ecology Letters* 3: 540-550.

Finney DJ, 1971. Probit analysis. Cambridge University Press, Cambridge. 333 pp.

Flury M (1996). Experimental evidence of transport of pesticides through field soils – a review. *Journal of Environmental Quality* 25: 25-45.

García de Llasera MP & Bernal-González M (2001). Presence of carbamate pesticides in environmental waters from the Northwest of Mexico: determination by liquid chromatography. *Water Research* 35: 1933-1940.

Guest RK, Ikehata K, El-Din MG, Smith DW (2006). Pesticides and Herbicides. *Water Environment Research* 78: 1755-1801.

Guilhermino L, Soares AMVM, Carvalho AP & Lopes MC (1998). Acute effects of 3,4-Dichloroaniline on blood of male Wistar rats. *Chemosphere* 37: 619-632.

Hanazato T (2001). Pesticide effects on freshwater zooplankton: an ecological perspective. *Environmental Pollution* 112: 1-10.

Heckmann L-H, Connon R, Hutchinson TH, Maund SJ, Sibly RM & Callaghan A (2006). Expression target and reference genes in *Daphnia magna* exposed to ibuprofen. *BMC Genomics* 7: 175.

Heckmann L-H, Bouetard A, Hill CJ, Sibly RM & Callaghan A (2007). A simple and rapid method for preserving RNA of aquatic invertebrates for ecotoxicogenomics. *Ecotoxicology* 16: 445-447.

Hintze KJ & Theil EC (2005). DNA and mRNA elements with complementary responses to hemin, antioxidant inducers, and iron control ferritin-L expression. *Proceedings of the National Academy of Sciences of the United States of America* 102: 15048-15052.

Hodgetts RB & O'Keefe SL (2006). Dopa decarboxylase: a model gene-enzyme system for studying development, behavior and systematics. *Annual Review of Entomology* 51: 259-284.

Huber A, Bach M & Frede HG (2000). Pollution of surface waters with pesticides in Germany: modelling non-point source inputs. *Agriculture, Ecosystems and Environment* 80: 191-204.

Ikenaka Y, Eun H, Ishizaka M & Miyabara Y (2006). Metabolism of pyrene by aquatic crustacean, *Daphnia magna*. *Aquatic Toxicology* 80: 158-165.

ISO (1996). Water quality-determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera: Crustacea) – acute toxicity test, ISO 66431. International Organisation for Standardisation, Geneva, Switzerland.

Janeway CA Jr. & Medzitov R (2002). Innate immune recognition. *Annual Review of Immunology* 20: 197-216.

Josephy PD (1997). Molecular Toxicology. New York: Oxford University Press. 336p.

Kato Y, Tokishita S, Ohta T & Yamagata H (2004). A vitellogenin chain containing a superoxide dismutase-like domain is the major component of yolk proteins in cladoceran crustacean *Daphnia magna*. *Gene* 334: 157-165.

Kimura S, Tokishita S, Ohta T Kobayashi M & Yamagata H (1999). Heterogeneity and differential expression under hypoxia of two-domain haemoglobin chains in the water flea, *Daphnia magna*. *Journal of Biological Chemistry* 274: 10649-10653.

Kobayashi M, Nezu T & Tanaka Y (1990). Hypoxic induction of hemoglobin synthesis in *Daphnia magna*. *Comparative Biochemistry and Physiology* A 97: 513-517.

Lampert W (2006). *Daphnia*: model herbivore, predator and prey. *Polish Journal of Ecology* 54: 607-620.

Larson SJ, Capel PD & Majewski MS (1998). Pesticides in surface waters – distribution, trends, and governing factors (Series: Pesticides in the hydrologic system; 3). Chelsea, Michigan: Ann Arbor Press. 373p.

Li W-m, Yin D-q, Zhou Y, Hu S-q & Wang LS (2003). 3,4-Dichloroaniline-induced oxidative stress in liver of crucian carp (*Carassius auratus*). *Ecotoxicology and Environmental Safety* 56: 251-255.

Little TJ, O'Connor B, Colegrave N, Watt K & Read AF (2003). Maternal transfer of strain-specific immunity. *Current Biology* 13: 489-492.

Malerba I, Castoldi AF, Parent-Massin D & Gribaldo L (2002). In vitro myelotoxicity of Propanil and 3,4-Dichloroaniline on Murine and Human CFU-E/BFU-E progenitors. *Toxicological Sciences* 69: 433-438.

McMillan DC, McRae TA & Hinson JA (1990). Propanil-induced methemoglobinemia and haemoglobin binding in the rat. *Toxicology and Applied Pharmacology* 105: 503-507.

Milatovic D, Gupta RC & Aschner M (2006). Anticholinesterase toxicity and oxidative stress. *The Scientific World JOURNAL* 6: 295-310.

Mitsou K, Koulianou A, Lambropoulou D, Pappas P, Albanis T & Lekka M (2006). Growth rate effects, responses of antioxidant enzymes and metabolic fate of the herbicide Propanil in the aquatic plant *Lemna minor*. *Chemosphere* 62: 275-284.

Moraes BS, Loro VL, Glusczac L, Pretto A, Menezes C, Marchezan E & Machado SE (2007). Effects of four rice herbicides on some metabolic and toxicology parameters of teleost fish (*Leporinus obtusidens*). *Chemosphere* 68: 1597-1601.

Muta T & Iwanaga S (1996). The role of hemolymph coagulation in innate immunity. *Current Opinion in Immunology* 8: 41-47.

Neumann NF & Galvez F (2002). DNA microarrays and toxicogenomics: applications for Ecotoxicology? *Biotechnology Advances* 20: 391-419.

Nunes F, Spiering D, Wolf M, Wendler A Pirow R & Paul RJ (2005). Sequence of Hemoglobin Gene 4 (*dmhb4*) and Southern Blot analysis provide evidence of more than four members of the *Daphnia magna* Globin family. *Bioscience, Biotechnology & Biochemistry* 69: 1193-1197.

OECD (2004). OECD guideline for testing of chemicals 202 - *Daphnia* sp., acute immobilisation test. Organization for the Economic Cooperation and Development. Paris.

Pastorelli R, Catenacci G, Guanci M, Fanelli R, Valotis E, Minoia C & Airoldi L (1998). 3,4-Dichloroaniline adducts in humans: preliminary data on agricultural workers exposed to propanil. *Biomarkers* 3: 227-233.

Pereira JL, Mendes CD & Gonçalves F (2007). Short- and long-term responses of *Daphnia* spp. to Propanil exposures in distinct food supply scenarios. *Ecotoxicology and Environmental Safety* 68: 386-396.

Pereira JL & Gonçalves (2007). Effects of food availability on the acute and chronic toxicity of the insecticide Methomyl to *Daphnia* spp. *The Science of the Total Environment* 386: 9-20.

Poynton HC, Varshavsky JR, Chang B, Cavigiolio G, Chan S, Holman PS, Loguinov AV, Bauer DJ, Komachi K, Theil EC, Perkins EJ, Hughes O & Vulpe CD (2007). *Daphnia magna* Ecotoxicogenomics provides mechanistic insights into metal toxicity. *Environmental Science & Technology* 41: 1044-1050.

Printes LB & Callaghan A (2004). A comparative study on the retationship between acetylcholinesterase activity and acute toxicity in *Daphnia magna* exposed to anticholinesterase insecticides. *Environmental Toxicology and Chemistry* 23: 1241-1247.

Quinn DM (1987). Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. *Chemical Reviews* 87: 955-979.

Reichenberger S, Bach M, Skitschak A & Frede H-G (2007). Mitigation strategies to reduce pesticide inputs into ground- and surface water and their effectiveness; a review. *The Science of the Total Environment* 384: 1-35.

Rider CV & LeBlanc GA (2006). Atrazine stimulated haemoglobin accumulation in *Daphnia magna*: is it hormonal or hypoxic? *Toxicological Sciences* 93: 443-449.

Robbens J, Van der Ven K, Maras M, Blust R & de Coen W (2007). Ecotoxicological risk assessment using DNA chips and cellular reporters. *TRENDS in Biotechnology* 25: 460-466.

Ruppert EE & Barnes RD (1996). Invertebrate Zoology, 6th ed. Saunders College Publishing: Fort Worth. 1056p.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V & Quackenbush J (2003). TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34(2): 374-378.

Schena M, Shalon D, Davis RW & Brown PO (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467-470.

Seidl MD, Paul RJ & Pirow R (2005). Effects of hypoxia acclimation on morpho-physiological traits over three generations of *Daphnia magna*. *The Journal of Experimental Biology* 208: 2165-2175.

Snape JR, Maund SJ, Pickford DB & Hutchinson TH (2004). Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial Ecotoxicology. *Aquatic Toxicology* 67: 143-154.

Soetaert A, Moens LN, Van der Ven K, Van Leemput K, Naudts B, Blust R & De Coen WM (2006). Molecular impact of propiconazole on *Daphnia magna* using a reproduction-related cDNA array. *Comparative Biochemistry and Physiology C* 142: 66-76.

Soetaert A, Vandenbroucke T, Van der Ven K, Maras M, Van Remortel P, Blust R & De Coen WM (2007). Molecular responses during Cadmium-induced stress in *Daphnia magna*: integration of differential gene expression with higher-level effects. *Aquatic Toxicology* 83: 212-222.

Tokishita S-I, Kato Y, Kobayashi T, Nakamura S, Ohta T & Yamagata H (2006). Organization and repression by juvenile hormone of a vitellogenin gene cluster in the crustacean, *Daphnia magna*. *Biochemical and Biophysical Research* 345: 362-370.

Stryer L (1999). Biochemistry, 4th ed. WH Freeman: New York. 1064p.

Terwilliger NB & Ryan M (2001). Ontogeny of crustacean respiratory proteins. *American Zoologist* 41: 1057-1067.

Tomlin CDS (2001). The pesticide manual. British Crop Protection Council: Surrey. 1260p.

Van Wijngaarden RPA, Brock TCM & van den Brink PJ (2005). Threshold levels for effects of insecticides in freshwater ecosystems: a review. *Ecotoxicology* 14: 355-380.

Villarroel MJ, Sancho E, Ferrando MD & Andreu E (2003). Acute, chronic and sub-lethal effects of the herbicide Propanil on *Daphnia magna*. *Chemosphere* 53: 857-864.

Watanabe H, Takahashi E, Nakamura Y, Oda S, Tatarazako N & Iguchi T (2007). Development of a *Daphnia magna* DNA microarray for evaluating the toxicity of environmental chemicals. *Environmental Toxicology and Chemistry* 26: 669-676.

Wendler F & Tooze S (2001). Syntaxin 6: the promiscuous behaviour of a SNARE protein. *Traffic* 2: 606-611.

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J& Speed TP (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Research 30 (4): e15.

Wilson PC & Foos JF (2006). Survey of carbamate and organophosphorous pesticide export from a South Florida (USA) agricultural watershed: implications of sampling frequency on ecological risk estimation. *Environmental Toxicology and Chemistry* 25: 2847-2852.

Zamponi GW (2003). Regulation of presynaptic calcium channels by synaptic proteins. *Journal of Pharmacological Sciences* 92: 79-83.

Zou E & Fingerman E (1997). Effects of estrogenic xenobiotics on molting of the water flea *Daphnia magna*. *Ecotoxicology and Environmental Safety* 38: 281-285.

APPENDIX V.I. Full list of BLAST homology search results for significantly differentially expressed gene sequences after exposure to Methomyl. 'Ratio' stands for the spot intensity ratio between treated and untreated (control) samples (see Material & Methods-*Bioinformatics* for details). For better handling of results, uninformative gene expression outputs such as unknown sequences and sequences with non-significant Blast homology (see Material & Methods-*Bioinformatics* for criteria) were removed from the dataset.

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Up-regulated genes				
IGU001_0024_B09	4.328	Clone JGIAZSN-5P22	*Daphnia pulex*	AC167694
WTH001_0005_O13	3.760	Cuticular protein	*D. melanogaster*	NM_137626
IGU001_0042_D09	3.666	12S ribosomal RNA gene	*Daphnia magna*	DQ116603
IGU001_0037_D05	3.599	Autophagy-specific gene 8a	*D. melanogaster*	NM_167245
WTH001_0013_B18	3.525	Cuticular protein 56F	*D. melanogaster*	NM_137626
IGU001_0038_H12	3.513	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0006_H02	3.415	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0013_K20	3.394	Cuticular protein 50Cb	*D. melanogaster*	NM_137057
IGU001_0009_A05	3.334	Ribosomal protein subunit 3	*C. sonorensis*	AY603568
IGU001_0054_B09	3.291	Vitellogenin fused with superoxide dismutase	*Daphnia magna*	AB252737
IGU001_0005_H08	3.286	Ferritin 3-like protein C	*Daphnia pulex*	DQ983433
IGU001_0034_B05	3.267	Mitochondrion	*Daphnia pulex*	DQ340821
IGU001_0020_F06	3.241	Mitochondrial genome	*Daphnia pulex*	*AF117817*
IGU001_0007_A10	3.215	dhb2 mRNA for haemoglobin	*Daphnia magna*	AB021136
WTH001_0005_C18	3.146	Cuticular protein 49Ag	*D. melanogaster*	NM_136932
IGU001_0004_E09	3.140	AF16 chromosome I	*C. briggsae*	XM_001669102
WTH001_0001_I05	3.120	Cytochrome oxidase subunit 1	*Daphnia magna*	DQ166849
WTH001_0004_F20	3.119	Similar to Tetraspanin 96F (Predicted)	*Apis mellifera*	XM_393020
IGU001_0009_C10	3.102	Selenoprotein 15	*A. gambiae*	AAL68777
IGU001_0015_D03	3.056	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0007_K01	3.054	Cuticular protein 78Cc	*D. melanogaster*	NM_141043
WTH001_0014_G09	3.026	Cuticular protein 74, RR-1 family	*A. gambiae*	XM_318987
RDE02	3.014	Similar to Tetraspanin 96F (Predicted)	*Apis mellifera*	XM_393020
WTH001_0010_L07	2.978	Insect pheromone-binding protein A10	*Apis mellifera*	CAJ01445
WTH001_0013_M15	2.957	Chitin deacetylase 1	*T. castaneum*	NM_001102476
IGU001_0027_G04	2.951	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0003_L18	2.946	Na,K-ATPase beta subunit	*Artemia* sp	X55780
IGU001_0004_H01	2.946	Chymotrypsin B	*P.vannamei*	Y10665
WTH001_0013_F21	2.931	Very low-density lipoprotein receptor	*Bombyx mori*	NM_001110325
WTH001_0014_N03	2.904	Chitin deacetylase 1	*T. castaneum*	NM_001102476
IGU001_0044_H07	2.886	16S ribosomal RNA	*Daphnia magna*	AY921452
IGU001_0044_H12	2.885	Mitochondrial genome	*Daphnia magna*	AF117817
IGU001_0007_G04	2.878	Ribosomal protein L7e	*Agriotes lineatus*	AM048999
WTH001_0010_I22	2.871	Chitin deacetylase 1	*T. castaneum*	NM_001102476
RAG08	2.844	Similar to Tetraspanin 96F (Predicted)	*Apis mellifera*	XM_393020
IGU001_0017_A12	2.826	Actin	*Apriona germari*	AY817141
IGU001_0011_B06	2.809	S6 mitochondrion	*Daphnia pulex*	DQ340839
WTH001_0012_J01	2.790	Cuticular protein 65Ax1	*D. melanogaster*	NM_001104053
WTH001_0004_K21	2.777	LDL receptor domain class A	*D. melanogaster*	AE003516
IGU001_0016_E04	2.744	Translationally-controlled tumor protein-like protein	*A.franciscana*	EU142261
IGU001_0016_G08	2.696	Carbonic anhydrase 1	*D. melanogaster*	NM_078837
IGU001_0013_C01	2.691	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0045_D09	2.664	S5e ribosomal protein	*D. cervinus*	AJ783868
WTH001_0013_G18	2.656	Chitin deacetylase 2	*T. castaneum*	NM_001102577
IGU001_0042_F12	2.632	60S ribosomal protein L23-like protein	*P. papatasi*	EU035821

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Up-regulated genes (Cont.)				
IGU001_0055_E05	2.624	Collagen, type I, alpha 1	*Xenopus laevis*	NM_001087352
IGU001_0012_D10	2.610	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0006_L07	2.590	Cuticular protein 65Ax1	*D. melanogaster*	NM_001104053
IGU001_0035_B10	2.581	Cuticular protein 65Ax1	*D. melanogaster*	NM_001104053
IGU001_0025_B03	2.569	Cuticular protein 74, RR-1 family	*A. gambiae*	XM_318987
IGU001_0002_E04	2.564	ATP synthase a chain	*Daphnia pulex*	NP_008625
WTH001_0003_D24	2.559	C-type lectin, galactose-binding	*A. gambiae*	XM_319374
IGU001_0048_G10	2.552	Cuticular protein 16, RR-1 family	*A. gambiae*	XM_315462
IGU001_0024_E07	2.496	Sulfotransferase	*Bombyx mori*	NM_001043537
IGU001_0038_B11	2.480	dhb3 mRNA for haemoglobin	*Daphnia magna*	AB021137
IGU001_0032_A08	2.462	Enolase	*Daphnia magna*	AAS02302
IGU001_0028_G03	2.425	Chitin deacetylase 2	*T. castaneum*	NM_001102577
WTH001_0010_A13	2.424	Cuticular protein 16, RR-1 family	*A. gambiae*	XM_315462
IGU001_0034_B04	2.409	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0001_H08	2.401	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0012_J23	2.394	Cuticular protein 78Cc	*D. melanogaster*	NM_141043
IGU001_0024_D05	2.389	Vitellogenin fused with superoxide dismutase	*Daphnia magna*	AB114859
IGU001_0018_E02	2.366	Cyclic AMP-regulated protein	*Bombyx mori*	NM_001046966
WTH001_0011_N01	2.360	Cuticular protein 56F	*D. melanogaster*	NM_137626
IGU001_0048_A10	2.342	Chitin deacetylase 2	*T. castaneum*	NM_001102577
IGU001_0041_E04	2.325	Cytochrome C oxidase subunit I,II,III; ATPase6,8	*Daphnia pulex*	U65669
WTH001_0007_G11	2.296	Alpha-amylase	*C. fluminea*	AF468016
WTH001_0003_F19	2.292	putative 60S ribosomal protein	*Flustra foliacea*	EU139203
IGU001_0040_C05	2.242	NADH dehydrogenase subunit 4	*T. dimidiata*	AF454699
WTH001_0012_P01	2.232	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
WTH001_0014_L06	2.209	Chitin deacetylase 1	*T. castaneum*	NM_001102476
WTH001_0009_J09	2.203	Cuticular protein 78Cc	*D. melanogaster*	NM_141043
IGU001_0012_A10	2.200	Ribosomal protein L37A	*D. melanogaster*	NM_164628
IGU001_0046_E08	2.185	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0009_N09	2.159	dhb2 mRNA for haemoglobin	*Daphnia magna*	AB021136
IGU001_0042_E03	2.158	Ribosomal protein L18	*D. melanogaster*	NM_139834
WTH001_0004_I15	2.143	Alpha-amylase (proximal)	*Drosophila orena*	D21129
IGU001_0017_A02	2.138	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0009_E08	2.124	Haemoglobin 4	*Daphnia magna*	AY737794
WTH001_0001_E24	2.092	Chymotrypsin 1; serine protease	*L. vannamei*	X66415
IGU001_0053_A05	2.085	Ribosomal protein subunit 3	*C. sonorensis*	AY603568
WTH001_0013_I08	2.080	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
IGU001_0022_F06	2.059	Putative preproadipsin	*Sus scrofa*	U29948
WTH001_0002_G11	2.053	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
R05CDR1E01	2.052	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
WTH001_0005_O15	2.016	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
IGU001_0036_H05	1.992	Cytochrome C oxidase subunit I, II	*Daphnia pulex*	U65669
IGU001_0041_A10	1.984	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0049_F06	1.974	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0006_C09	1.974	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0004_J21	1.931	Cuticle protein (Wcp9)	*Bombyx mori*	NM_001043404
WTH001_0009_E17	1.929	Cuticular protein 50Cb	*D. melanogaster*	NM_137057
WTH001_0012_C23	1.909	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
IGU001_0031_C11	1.906	Mitochondrion	*Daphnia pulex*	DQ340832
WTH001_0010_I09	1.902	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
IGU001_0030_A02	1.898	Collagen alpha-1 chain	*L. salmonis*	EF490833
IGU001_0040_G05	1.888	TRE genomic sequence	*Daphnia obtusa*	EF077786
IGU001_0018_B04	1.875	LIN NADH dehydrogenase-like gene	*Daphnia pulex*	EF077791
IGU001_0040_H09	1.871	dhb3 mRNA for haemoglobin	*Daphnia magna*	AB021137

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Up-regulated genes (Cont.)				
WTH001_0002_O10	1.865	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
WTH001_0012_D01	1.840	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0009_M13	1.836	Vitellogenin 1	*Daphnia magna*	BAD05137
IGU001_0025_B06	1.825	Insect cuticle protein (Chitin_bind_4)	*T. castaneum*	XP_969336
R16LFR1C08	1.805	Cuticular protein 65Ax1	*D. melanogaster*	NM_001104053
IGU001_0022_F01	1.800	Zinc proteinase Mpc1	*L. vannamei*	DQ398567
WTH001_0011_H08	1.797	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0002_G22	1.781	Cuticle protein (Wcp9)	*Bombyx mori*	NM_001043404
IGU001_0056_B06	1.773	Dehydrogenase	*Aedes aegypti*	DQ440305
WTH001_0010_G06	1.756	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
IGU001_0052_E05	1.755	Serine collagenase 1 precursor	*Celuca pugilator*	U49931
IGU001_0023_A09	1.693	Histone H4 (H4) gene	*Mytilus edulis*	AY267754
WTH001_0001_A18	1.688	16S ribosomal RNA gene	*Daphnia magna*	AY921452
IGU001_0011_B08	1.684	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0001_G24	1.678	Rhodopsin (Rh1)	*N. oerstedii*	DQ646869
IGU001_0016_B11	1.654	S11 mitochondrion	*Daphnia pulex*	DQ340832
RRH03	1.645	Chemosensory protein	*Daphnia pulex*	DQ855481
WTH001_0013_E12	1.636	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
IGU001_0004_D06	1.636	NADH dehydrogenase subunit 2	*D. melanogaster*	AE003579
WTH001_0004_B24	1.624	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
IGU001_0031_E06	1.612	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0030_D08	1.604	40S ribosomal protein S19	*O. parkeri*	EF633859
IGU001_0042_F06	1.592	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0046_F02	1.577	Ribosomal protein rpl31	*E. complanata*	EU125025
WTH001_0001_L16	1.564	Cytochrome oxidase subunit 1	*Daphnia magna*	DQ166849
R09CAF1F04	1.559	Cuticular protein 49Aa	*D. melanogaster*	NM_001103815
IGU001_0014_E11	1.558	Cytochrome C oxidase subunit I	*Daphnia pulex*	U65669
WTH001_0003_F07	1.555	Chitin-binding domain type 2	*Aedes aegypti*	EAT45952
WTH001_0010_L20	1.542	Putative calcium-binding protein p22	*M. hirsutus*	EF070534
WTH001_0001_I11	1.537	Trypsin	*Aplysina fistularis*	AF486488
R16LFR1H06	1.512	Cuticular protein 16, RR-1 family	*A. gambiae*	XM_315462
IGU001_0052_G08	1.510	Putative ribosomal protein S14e	*Diaphorina citri*	DQ673411
IGU001_0040_C04	1.509	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0012_P21	1.480	Phosphopantothenoylcysteine synthetase	*X. tropicalis*	NM_001079164
IGU001_0034_B08	1.462	Ribosomal protein S12	*A. franciscana*	EF660898
IGU001_0019_F03	1.446	Putative ribosomal protein L24e	*A. pisum*	DQ413195
R10CAR1B12	1.445	Cuticular protein 56F	*D. melanogaster*	NM_137626
IGU001_0025_H06	1.436	Cuticular protein 16, RR-1 family	*A. gambiae*	XM_315462
R01CDF1G03	1.434	Cuticular protein 12, RR-1 family	*A. gambiae*	XM_315456
IGU001_0005_G10	1.428	Nucleosome assembly protein 1-like 1	*Xenopus laevis*	NM_001087078
WTH001_0003_J03	1.422	Carboxypeptidase T06A4.1b	*C. elegans*	NM_182009
R11CAR2C01	1.420	Cuticular protein 49Ah	*D. melanogaster*	NM_136933
IGU001_0014_D05	1.417	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0017_F08	1.383	Chymotrypsin 1	*P.vanameii*	X66415
WTH001_0006_J08	1.383	Similar to angiomotin (predicted)	*N. vitripennis*	XM_001604449
IGU001_0004_B04	1.380	Ferritin	*Daphnia pulex*	AJ245734
WTH001_0005_I16	1.357	Oxidative stress protein	*Aurelia aurita*	AY836662
WTH001_0007_M11	1.354	Gasp precursor	*D. melanogaster*	AF070734
WTH001_0009_O07	1.313	Pros45 proteosome subunit homolog	*D. melanogaster*	AF043734
IGU001_0016_E08	1.308	Chloride/bicarbonate anion exchanger	*A. gambiae*	AY280611
WTH001_0004_A22	1.301	Serine/threonine-protein kinase pim-3	*Homo sapiens*	BC052239
IGU001_0024_G05	1.279	16S ribosomal RNA gene	*Daphnia magna*	AY921452
IGU001_0052_G09	1.258	Thymosin	*Bombyx mori*	NM_001047021
WTH001_0003_N08	1.257	Similar to WD repeat protein 26 (predicted)	*N. vitripennis*	XM_001604825

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Up-regulated genes (Cont.)				
IGU001_0042_B08	1.255	Peritrophic membrane chitin binding protein 2	*Trichoplusia ni*	AY345125
IGU001_0017_D09	1.255	4 ferritin 3-like protein C	*Daphnia pulex*	DQ983433
R01CDF1G12	1.250	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
WTH001_0003_D10	1.231	Enolase	*Daphnia magna*	AY522935
WTH001_0005_C20	1.230	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
WTH001_0003_N24	1.224	18S rRNA gene	*Daphnia magna*	AM490278
WTH001_0014_C12	1.220	C-type lectin, galactose-binding	*A. gambiae*	XM_319371
WTH001_0002_F15	1.204	Similar to Ghitm-prov protein (predicted)	*N. vitripennis*	XM_001608077
R01CDF1D08	1.202	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
WTH001_0007_H08	1.202	Zinc proteinase Mpc1	*L. vannamei*	DQ398567
IGU001_0002_A01	1.187	16S ribossomal RNA	*Daphnia magna*	AY921452
WTH001_0013_L07	1.159	C-type lectin, galactose-binding	*A. gambiae*	XM_319371
IGU001_0033_C07	1.155	16S ribosomal RNA gene	*Daphnia magna*	AY921452
IGU001_0041_D03	1.152	Arginine kinase	*L. vannamei*	EU346737
WTH001_0003_C07	1.150	Cytochrome C oxidase subunit I	*Daphnia pulex*	U65669
WTH001_0013_N20	1.143	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
IGU001_0036_F09	1.113	S25e ribosomal protein	*P. albinus*	AJ783896
IGU001_0025_E12	1.112	Cuticular protein 49Ae	*D. melanogaster*	NM_136930
WTH001_0011_C05	1.109	Gasp precursor	*D. melanogaster*	AF070734
IGU001_0041_F03	1.093	Trypsin	*Aplysina fistularis*	AF486488
IGU001_0024_F05	1.087	Mitochondrial genome	*Daphnia magna*	AF117817
IGU001_0040_D09	1.061	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0007_E07	1.060	Similar to zinc finger	*T. castaneum*	XM_961710
WTH001_0004_E24	1.056	Larval cuticle protein 12.3	*Apriona germari*	AAM66718
WTH001_0010_M01	1.043	Putative muscular protein20	*M. hirsutus*	EF070497
R18IBF2A03	1.032	Cuticule extracellular matrix structural constituent	*A. gambiae*	AAAB01008980
WTH001_0013_K10	1.021	Cuticle protein 12.3	*Apriona germari*	AF518323
IGU001_0021_H04	1.015	Cytochrome C oxidase subunit I	*Daphnia pulex*	U65669
IGU001_0017_B11	1.014	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0001_F10	1.010	Ribosomal protein S26	*Plutella xylostella*	AB180410
WTH001_0001_L03	1.000	ribosomal protein 13	*Lonomia obliqua*	AY829770
Down-regulated genes				
WTH001_0001_I21	-2.381	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
IGU001_0014_F05	-2.298	Receptor expression-enhancing protein 5	*Danio rerio*	AAH59545
IGU001_0015_D12	-2.242	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0032_H11	-2.231	GM2 ganglioside activator protein	*Xenopus laevis*	AAH74424
IGU001_0033_A12	-2.213	Ubc D1 mRNA for ubiquitin-conjugating enzyme	*D. melanogaster*	X62575
IGU001_0007_G01	-2.183	Cytochrome c oxidase subunit 1	*Daphnia pulex*	NP_008622
IGU001_0013_A05	-2.141	dhb3 mRNA for haemoglobin	*Daphnia magna*	AB021137
IGU001_0050_B09	-2.101	Putative peptidyl-prolyl cis-trans isomerase E	*M. hirsutus*	EF070482
IGU001_0021_H07	-2.099	Mitochondrion	*Daphnia pulex*	DQ340839
IGU001_0056_E06	-2.089	Ribosomal protein S25	*P. albinus*	CAH04344
IGU001_0030_G11	-2.066	Ribosomal protein L9	*A. franciscana*	ABC02755
WTH001_0003_I15	-2.061	Trypsin	*Aplysina fistularis*	AAO12215
IGU001_0043_F09	-2.057	16S ribosomal RNA gene	*Daphnia magna*	DQ470575
IGU001_0045_A09	-2.049	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0004_F08	-2.048	Mitochondrion	*Daphnia pulex*	DQ340825
WTH001_0004_L21	-2.008	Extracellular cyanophycinase (cphE)	*P. anguilliseptica*	AY065671
WTH001_0009_B13	-2.006	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0012_G09	-2.000	GM2 ganglioside activator protein	*Danio rerio*	AAH92784
IGU001_0013_F02	-1.999	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0014_K09	-1.988	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0054_F02	-1.988	Vesicle coat complex COPII GTPase subunit	*Aedes aegypti*	DQ440264

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Down-regulated genes (Cont.)				
IGU001_0024_G02	-1.987	Acidic p0 ribosomal protein	*D. cervinus*	AJ783862
IGU001_0020_B04	-1.987	Mitochondrion	*Daphnia pulex*	DQ340820
WTH001_0014_D22	-1.986	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0009_H14	-1.977	Cuticle protein 7	*Tenebrio molitor*	CAA03880
WTH001_0005_G08	-1.968	Carboxylesterase	*S. exigua*	EF580101
WTH001_0014_K24	-1.964	Cuticle protein 7	*Blaberus craniifer*	P82120
IGU001_0043_C02	-1.946	Ribosomal RNA gene	*Daphnia magna*	AY921452
WTH001_0001_J23	-1.944	18S rRNA gene	*Daphnia magna*	AM490278
WTH001_0013_B24	-1.940	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0003_L22	-1.939	Strain S2 mitochondrion	*Daphnia pulex*	DQ340842
WTH001_0012_P06	-1.934	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0002_N09	-1.931	Cuticle protein 7	*Blaberus craniifer*	P82120
IGU001_0020_E06	-1.923	Thymosin	*Bombyx mori*	NM_001110348
IGU001_0038_H10	-1.922	Ribosomal protein S20	*O. mykiss*	CAC44156
IGU001_0020_B05	-1.919	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0002_B18	-1.916	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0051_H06	-1.902	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0020_D04	-1.877	Mitochondrion	*Daphnia pulex*	DQ340832
IGU001_0031_D12	-1.865	39 ribosome-associated membrane protein	*O. fasciatus*	EF382747
IGU001_0031_D07	-1.857	Cytochrome b	*Daphnia pulex*	NP_008632
WTH001_0010_K18	-1.854	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0014_E03	-1.843	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0003_A23	-1.809	Ribosomal protein L28	*Sphaerius sp.*	CAJ17404
WTH001_0003_I08	-1.778	Ferritin 3-like protein C	*Daphnia pulex*	DQ983433
WTH001_0001_M08	-1.742	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0017_B10	-1.741	Chymotrypsin B2	*L. vannamei*	CAA71673
IGU001_0044_D10	-1.734	18S rRNA gene	*Daphnia magna*	AM490278
WTH001_0007_E08	-1.722	Similar to doughnut on 2 CG17559-PA (predicted)	*Apis mellifera*	XM_393673
WTH001_0001_J11	-1.706	DEAD-box RNA-dependent helicase p68	*C. auratus*	AY821682
WTH001_0007_L07	-1.700	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0005_P10	-1.669	Similar to apolipoprotein D (predicted)	*S. purpuratus*	XM_001181564
WTH001_0010_P23	-1.592	Cuticle protein 7	*Blaberus craniifer*	P82120
WTH001_0004_P21	-1.592	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0018_B12	-1.534	ATP synthase a chain	*Daphnia pulex*	Q95782
WTH001_0009_N04	-1.523	PHB depolymerase	*Acidovorax sp.*	AB015309
IGU001_0019_C08	-1.476	Ribosomal protein S19	*A. monolakensis*	DQ886792
IGU001_0019_B07	-1.468	Mitochondrion	*Daphnia pulex*	DQ340832
WTH001_0012_N18	-1.459	Ecdysteroid-regulated protein	*L. vannamei*	DQ398569
IGU001_0028_H07	-1.416	Cuticle structural protein post-ecdysial	*Tenebrio molitor*	Q7M4D9
WTH001_0001_G20	-1.409	Similar to adenylosuccinate synthetase (predicted)	*Bos taurus*	NM_001099192
WTH001_0009_L11	-1.408	Lipoprotein N-terminal Domain	*Aedes aegypti*	EAT39606
WTH001_0007_I13	-1.390	Ecdysteroid-regulated protein	*L. vannamei*	DQ398569
IGU001_0011_E08	-1.384	Obstractor D	*T. castaneum*	NM_001080099
WTH001_0012_I23	-1.382	Ecdysteroid-regulated protein	*L. vannamei*	DQ398569
WTH001_0005_K21	-1.381	Fatty acid binding protein	*S. japonicum*	L23322
IGU001_0002_C09	-1.361	Cytochrome b	*Daphnia pulex*	ABD19355
WTH001_0001_N15	-1.358	Epididymal secretory protein E1 precursor	*D. melanogaster*	Q9VQ62
WTH001_0001_M13	-1.352	Chitin binding domain-containing protein	*A. franciscana*	EU072032
IGU001_0020_D03	-1.345	Palmitoyl-protein thioesterase 2	*Bos taurus*	NM_001035318
WTH001_0012_A01	-1.337	Chymotrypsin 1	*L. vannamei*	X66415
WTH001_0005_I15	-1.333	Fatty acid binding protein	*S. japonicum*	L23322
WTH001_0007_M04	-1.319	Small nuclear ribonucleoprotein D3	*Mus musculus*	NM_026095
IGU001_0002_E11	-1.312	Mitochondrion	*Daphnia pulex*	DQ340832
WTH001_0001_D16	-1.306	CytC oxidase; ATPase8,6; NADH dehydrogenase	*Daphnia pulex*	U65669

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Down-regulated genes (Cont.)				
WTH001_0013_I13	-1.299	Putative preproadipsin	*Sus scrofa*	U29948
IGU001_0023_A03	-1.289	Peptidyl-prolyl cis-trans isomerase	*A. subalbatus*	EU207997
WTH001_0012_H23	-1.253	Fatty acid binding protein	*S. japonicum*	L23322
WTH001_0013_F20	-1.241	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0003_H02	-1.232	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0025_B11	-1.231	Haemoglobin 3	*Daphnia magna*	AB021137
WTH001_0010_L05	-1.220	Putative preproadipsin	*Sus scrofa*	U29948
RRD03	-1.208	Serine-type protease inhibitor	*B. microplus*	CAC82583
WTH001_0009_F20	-1.206	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0009_O15	-1.204	Cuticle structural protein post-ecdysial	*Tenebrio molitor*	S78003
IGU001_0006_A12	-1.200	Cuticle protein	*D. melanogaster*	U84747
IGU001_0014_G11	-1.199	Annexin B10C	*A. gambiae*	XM_310252
IGU001_0021_A10	-1.197	Heat shock protein HSP90	*D. klunzingeri*	Y17848
IGU001_0007_E10	-1.190	16S ribosomal RNA gene	*Daphnia magna*	AY921452
WTH001_0009_J04	-1.183	CLIP-domain serine protease subfamily D	*A. gambiae*	XM_317284
WTH001_0007_C20	-1.174	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0011_I11	-1.162	GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase	*D. melanogaster*	NM_137890
WTH001_0011_J17	-1.145	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0005_L07	-1.140	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0009_C09	-1.131	Mitochondrial ribosomal protein L2 (nuclear gene)	*Bombyx mori*	NM_001044151
WTH001_0001_N05	-1.105	Elongation factor 1 alpha	*Bombyx mori*	NM_001044045
WTH001_0003_O03	-1.096	Arginine kinase	*L. polyphemus*	U09809
IGU001_0036_G10	-1.091	Rab-protein 1	*D. melanogaster*	NM_169953
IGU001_0051_B05	-1.072	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0003_C20	-1.070	16S ribosomal RNA	*Daphnia magna*	AY921452
IGU001_0043_D05	-1.064	Putative Rab7 mRNA	*O. nigricans*	AY725788
WTH001_0013_N12	-1.059	Translation initiation factor	*A. subalbatus*	EU205638
IGU001_0009_D11	-1.055	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0045_F02	-1.054	Similar to protein phosphatase 1K (predicted)	*R. norvegicus*	NM_001107863
IGU001_0044_E10	-1.045	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0004_G22	-1.044	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
IGU001_0046_E12	-1.031	Putative 60S ribosomal protein	*Flustra foliacea*	EU139224
WTH001_0005_B04	-1.027	Alcohol dehydrogenase class 3	*B. floridae*	AF154331
WTH001_0002_G18	-1.025	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
IGU001_0011_D08	-1.012	Ribosomal protein S11-2	*Bombyx mori*	AY769326
IGU001_0006_D10	-1.012	Ribosomal protein S17	*Diaphorina citri*	ABG81965

FULL LIST OF SPECIES MATCHED IN HOMOLOGY SEARCH:

Vertebrates – *Bos taurus*; *Carassius auratus; Danio rerio*; *Homo sapiens*; *Mus musculus*; *Oncorhynchus mykiss*; *Rattus norvegicus*; *Sus scrofa*; *Xenopus laevis*; *Xenopus tropicalis*.

Invertebrates – *Acyrthosiphon pisum*; *Aedes aegypti*; *Agriotes lineatus*; *Anopheles gambiae*; *Apis mellifera*; *Aplysina fistularis*; *Apriona germari*; *Argas monolakensis*; *Armigeres subalbatus*; *Artemia* sp.; *Artemia franciscana*; *Aurelia aurita*; *Blaberus craniifer; Bombyx mori*; *Boophilus microplus*; *Branchiostoma floridae*; *Caenorhabditis briggsae*; *Caenorhabditis elegans*; *Celuca pugilator*; *Corbicula fluminea*; *Culicoides sonorensis*; *Daphnia magna*; *Daphnia pulex*; *Daphnia obtuse*; *Dascillus cervinus*; *Dendronephthya klunzingeri*; *Diaphorina citri*; *Drosophila orena*; *Drosophila melanogaster*; *Eurythoe complanata; Flustra foliacea*; *Lepeophtheirus salmonis*; *Limulus polyphemus*; *Litopenaeus vannamei*; *Lonomia obliqua*; *Maconellicoccus hirsutus*; *Mytilus edulis*; *Nasonia vitripennis*; *Neogonodactylus oerstedii*; *Oncometopia nigricans*; *Oncopeltus fasciatus*; *Ornithodoros parkeri*; *Penaeus vanamei*; *Phlebotomus papatasi*; *Platystomos albinus*; *Plutella xylostella*; *Portunus pelagicus*; *Schistosoma japonicum*; *Sphaerius* sp.; *Spodoptera exigua*; *Strongylocentrotus purpuratus*; *Tenebrio molitor*; *Triatoma dimidiata*; *Tribolium castaneum*; *Trichoplusia ni*

Bacteria – *Pseudomonas anguilliseptica*; *Acidovorax* sp.

APPENDIX V.II. Full list of BLAST homology search results for significantly differentially expressed gene sequences after exposure to Propanil. 'Ratio' stands for the spot intensity ratio between treated and untreated (control) samples (see Material & Methods-*Bioinformatics* for details). For better handling of results, uninformative gene expression outputs such as unknown sequences and sequences with non-significant Blast homology (see Material & Methods-*Bioinformatics* for criteria) were removed from the dataset.

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Up-regulated genes				
IGU001_0037_D05	5.038	Autophagy-specific gene 8a	*D. melanogaster*	NM_167245
IGU001_0042_D09	4.975	12S ribosomal RNA gene	*Daphnia magna*	DQ116603
IGU001_0006_H02	4.882	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0038_H12	4.771	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0004_F20	4.056	Similar to Tetraspanin 96F (Predicted)	*Apis mellifera*	XM_393020
RDE02	3.930	Similar to Tetraspanin 96F (Predicted)	*Apis mellifera*	XM_393020
IGU001_0030_A07	3.699	Ferritin 1-like protein A	*Daphnia pulex*	DQ983438
IGU001_0034_B05	3.663	Mitochondrion	*Daphnia pulex*	DQ340821
WTH001_0009_E08	3.513	Haemoglobin 4	*Daphnia magna*	AY737794
RAG08	3.467	Similar to Tetraspanin 96F (Predicted)	*Apis mellifera*	XM_393020
IGU001_0044_H12	3.347	Mitochondrial genome	*Daphnia magna*	AF117817
WTH001_0001_H08	3.183	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0004_G03	3.148	collagen alpha-1 chain	*L. salmonis*	EF490833
WTH001_0004_I15	3.111	Alpha-amylase (proximal)	*Drosophila orena*	D21129
WTH001_0007_G11	3.073	Alpha-amylase	*C. fluminea*	AF468016
IGU001_0014_E11	3.055	Cytochrome C oxidase subunit I	*Daphnia pulex*	U65669
IGU001_0029_E10	2.983	Mitochondrion	*Daphnia magna*	DQ340832
IGU001_0032_A08	2.953	Enolase	*Daphnia magna*	AAS02302
IGU001_0034_B04	2.953	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0003_D24	2.940	C-type lectin, galactose-binding	*A. gambiae*	XM_319374
IGU001_0023_A09	2.911	Histone H4 (H4) gene	*Mytilus edulis*	AY267754
IGU001_0034_B08	2.893	Ribosomal protein S12	*A. franciscana*	EF660898
IGU001_0017_A02	2.842	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0024_D05	2.767	Vitellogenin fused with superoxide dismutase	*Daphnia magna*	AB114859
WTH001_0001_I05	2.665	Cytochrome oxidase subunit 1	*Daphnia magna*	DQ166849
IGU001_0040_C05	2.621	NADH dehydrogenase subunit 4	*T. dimidiata*	AF454699
IGU001_0022_F12	2.580	40S ribosomal protein S19	*O. parkeri*	EF633859
IGU001_0011_B08	2.550	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0014_N03	2.487	Chitin deacetylase 1	*T. castaneum*	NM_001102476
WTH001_0014_H16	2.440	dhb3 mRNA for haemoglobin	*Daphnia magna*	AB021137
WTH001_0011_J08	2.437	dhb3 mRNA for haemoglobin	*Daphnia magna*	AB021137
WTH001_0013_M15	2.414	Chitin deacetylase 1	*T. castaneum*	NM_001102476
WTH001_0013_F21	2.431	Very low-density lipoprotein receptor	*Bombyx mori*	NM_001110325
IGU001_0016_G08	2.389	Carbonic anhydrase 1	*D. melanogaster*	NM_078837
IGU001_0040_H09	2.389	dhb3 mRNA for haemoglobin	*Daphnia magna*	AB021137
IGU001_0022_F01	2.382	Zinc proteinase Mpc1	*L. vannamei*	DQ398567
IGU001_0039_H08	2.331	Syntaxin 6 CG7736-RD	*D. melanogaster*	NM_206092
WTH001_0004_K21	2.233	LDL receptor domain class A	*D. melanogaster*	AE003516
WTH001_0009_M13	2.232	Vitellogenin 1	*Daphnia magna*	BAD05137
IGU001_0018_B04	2.218	LIN NADH dehydrogenase-like gene	*Daphnia pulex*	EF077791
IGU001_0020_A10	2.205	Ubiquitin-like/S30 ribosomal fusion protein	*L. testaceipes*	AY961508
WTH001_0002_G11	2.173	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0012_P01	2.142	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
WTH001_0005_A13	2.134	Putative preproadipsin	*Sus scrofa*	U29948
WTH001_0010_I22	2.133	Chitin deacetylase 1	*T. castaneum*	NM_001102476

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Up-regulated genes (Cont.)				
IGU001_0046_F02	2.121	Ribosomal protein rpl31	*E. complanata*	EU125025
IGU001_0024_G05	2.119	16S ribosomal RNA gene	*Daphnia magna*	AY921452
IGU001_0030_A02	2.099	Collagen alpha-1 chain	*L. salmonis*	EF490833
WTH001_0010_I14	2.035	Haemoglobin 3	*Daphnia magna*	AB021137
WTH001_0014_L06	2.026	Chitin deacetylase 1	*T. castaneum*	NM_001102476
WTH001_0006_C09	1.996	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0013_G18	1.964	Chitin deacetylase 2	*T. castaneum*	NM_001102577
WTH001_0004_K03	1.959	Haemoglobin gene cluster (dhb3, dhb1 and dhb2)	*Daphnia magna*	AB021134
WTH001_0013_I13	1.957	Putative preproadipsin	*Sus scrofa*	U29948
WTH001_0013_I08	1.954	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
WTH001_0001_D16	1.927	CytC oxidase; ATPase8,6; NADH dehydrogenase	*Daphnia pulex*	U65669
WTH001_0011_A20	1.927	dhb2 mRNA for haemoglobin	*Daphnia magna*	AB021136
WTH001_0002_O10	1.911	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
WTH001_0005_D21	1.892	dhb2 mRNA for haemoglobin	*Daphnia magna*	AB021136
IGU001_0048_A10	1.891	Chitin deacetylase 2	*T. castaneum*	NM_001102577
IGU001_0028_G03	1.890	Chitin deacetylase 2	*T. castaneum*	NM_001102577
WTH001_0011_C23	1.881	Haemoglobin gene cluster (dhb3, dhb1 and dhb2)	*Daphnia magna*	AB021134
IGU001_0050_H05	1.874	Cytochrome oxidase subunit I	*Daphnia magna*	AY803046
WTH001_0011_H08	1.873	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
IGU001_0042_F06	1.867	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0016_B11	1.816	S11 mitochondrion	*Daphnia pulex*	DQ340832
WTH001_0010_G06	1.808	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0012_D01	1.780	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0011_G13	1.777	dhb2 mRNA for haemoglobin	*Daphnia magna*	AB021136
WTH001_0011_J17	1.764	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0009_H22	1.759	Cystatin precursor	*T. tridentatus*	Q7M429
WTH001_0005_O15	1.757	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0001_B08	1.752	Ubiquitin carboxyl-terminal esterase L4	*X. tropicalis*	NM_001017120
IGU001_0036_G10	1.751	Rab-protein 1	*D. melanogaster*	NM_169953
IGU001_0020_B02	1.749	4 ferritin 3-like protein C	*Daphnia pulex*	DQ983433
WTH001_0013_E12	1.702	Structural constituent of peritrophic membrane	*A. subalbatus*	EU206887
WTH001_0010_I09	1.700	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0010_L05	1.699	Putative preproadipsin	*Sus scrofa*	U29948
IGU001_0003_A05	1.693	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0035_F02	1.678	16S ribosomal RNA	*Daphnia magna*	AY921452
WTH001_0011_D04	1.661	Mitochondrial genome	*Daphnia magna*	AF117817
IGU001_0007_C11	1.658	Vang-like protein 2	*Danio rerio*	BC065983
IGU001_0031_E06	1.655	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0051_B05	1.632	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0001_L16	1.631	Cytochrome oxidase subunit 1	*Daphnia magna*	DQ166849
IGU001_0041_F03	1.619	Trypsin	*Aplysina fistularis*	AF486488
WTH001_0010_L20	1.611	Putative calcium-binding protein p22	*M. hirsutus*	EF070534
WTH001_0006_N17	1.591	dhb2 mRNA for haemoglobin	*Daphnia magna*	AB021136
WTH001_0013_L21	1.586	Serine protease	*P. leniusculus*	AAX55746
IGU001_0040_A10	1.579	16S ribosomal RNA	*Daphnia magna*	AY921452
WTH001_0010_B07	1.542	Haemoglobin 1	*Daphnia magna*	U67067
IGU001_0019_F03	1.536	Putative ribosomal protein L24e	*A. pisum*	DQ413195
WTH001_0014_O13	1.527	Haemoglobin 2	*Daphnia magna*	AB021136
IGU001_0009_C10	1.524	Selenoprotein 15	*A. gambiae*	AAL68777
IGU001_0036_H05	1.509	Cytochrome C oxidase subunit I, II	*Daphnia pulex*	U65669
WTH001_0003_C07	1.507	Cytochrome C oxidase subunit I	*Daphnia pulex*	U65669
WTH001_0001_I11	1.502	Trypsin	*Aplysina fistularis*	AF486488
WTH001_0004_G22	1.490	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0007_O01	1.481	Glutamine synthetase	*A. franciscana*	EU072033

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Up-regulated genes (Cont.)				
IGU001_0014_B08	1.462	Ribosomal protein S13	*S. senegalensis*	AB291566
IGU001_0039_E06	1.446	Trypsin	*A. fistularis*	AF486488
WTH001_0014_C12	1.429	C-type lectin, galactose-binding	*A. gambiae*	XM_319371
WTH001_0005_N18	1.416	Acyl-CoA desaturase HassGATD	*H. assulta*	AF482905
WTH001_0001_P08	1.411	16S ribosomal RNA	*Daphnia magna*	AY921452
WTH001_0007_G12	1.409	Haemoglobin 1	*Daphnia magna*	U67067
WTH001_0010_F06	1.408	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
IGU001_0016_C12	1.405	Ribosomal protein S30	*L. testaceipes*	AY961508
IGU001_0013_D10	1.379	Metalloproteinase 2	*Hydra vulgaris*	AF140020
WTH001_0002_G18	1.371	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0003_P01	1.338	Dopa decarboxylase	*A. subalbatus*	EU207483
IGU001_0002_E04	1.331	ATP synthase a chain	*Daphnia pulex*	NP_008625
WTH001_0010_L17	1.325	Ornithine decarboxylase	*Bombyx mori*	NM_001046992
WTH001_0003_L17	1.313	Cytochrome c oxidase subunit 1	*Daphnia pulex*	NP_008622
WTH001_0009_N09	1.310	dhb2 mRNA for haemoglobin	*Daphnia magna*	AB021136
WTH001_0003_D10	1.277	Enolase	*Daphnia magna*	AY522935
WTH001_0013_L07	1.246	C-type lectin, galactose-binding	*A. gambiae*	XM_319371
WTH001_0003_J03	1.244	Carboxypeptidase T06A4.1b	*C. elegans*	NM_182009
WTH001_0004_A22	1.243	Serine/threonine-protein kinase pim-3	*Homo sapiens*	BC052239
WTH001_0007_E07	1.242	Similar to zinc finger	*T. castaneum*	XM_961710
IGU001_0002_F10	1.235	Myosin light chain	*A. franciscana*	EF660908
WTH001_0001_L03	1.226	Ribosomal protein 13	*Lonomia obliqua*	AY829770
WTH001_0005_I16	1.160	Oxidative stress protein	*Aurelia aurita*	AY836662
IGU001_0023_A07	1.159	Alpha-amylase	*C. fluminea*	AF468016
WTH001_0013_K10	1.145	Cuticle protein 12.3	*Apriona germari*	AF518323
IGU001_0011_D08	1.142	Ribosomal protein S11-2	*Bombyx mori*	AY769326
WTH001_0009_M06	1.134	Similar to tetraspanin family protein (predicted)	*S. purpuratus*	XM_001178552
WTH001_0006_H03	1.107	Large subunit ribosomal RNA	*Daphnia magna*	AF346515
IGU001_0016_H10	1.104	Mitochondrion	*Daphnia pulex*	DQ340832
WTH001_0014_H04	1.096	Haemoglobin (Dhb1)	*Daphnia magna*	U67067
WTH001_0001_G24	1.093	Rhodopsin (Rh1)	*N. oerstedii*	DQ646869
IGU001_0041_D03	1.065	Arginine kinase	*L. vannamei*	EU346737
IGU001_0052_G08	1.061	Putative ribosomal protein S14e	*Diaphorina citri*	DQ673411
IGU001_0022_B01	1.045	Innexin 2	*Bombyx mori*	EF197891
WTH001_0012_F13	1.044	NFkB protein	*S. purpuratus*	NM_214654
IGU001_0005_G10	1.042	Nucleosome assembly protein 1-like 1	*Xenopus laevis*	NM_001087078
WTH001_0012_P21	1.033	Phosphopantothenoylcysteine synthetase	*X. tropicalis*	NM_001079164
WTH001_0013_B21	1.029	Haemoglobin 1	*Daphnia magna*	U67067
IGU001_0042_B12	1.017	Similar to katanin 60 CG10229-PA (predicted)	*Apis mellifera*	XM_397402
Down-regulated genes				
WTH001_0002_B18	-3.465	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0003_I15	-3.388	Trypsin	*Aplysina fistularis*	AAO12215
WTH001_0003_I08	-3.344	Ferritin 3-like protein C	*Daphnia pulex*	DQ983433
WTH001_0009_H14	-3.316	Cuticle protein 7	*Tenebrio molitor*	CAA03880
WTH001_0001_J23	-3.307	18S rRNA gene	*Daphnia magna*	AM490278
WTH001_0009_B13	-3.293	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0002_N09	-3.284	Cuticle protein 7	*Blaberus craniifer*	P82120
WTH001_0003_L22	-3.269	Strain S2 mitochondrion	*Daphnia pulex*	DQ340842
WTH001_0010_K18	-3.237	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0004_F08	-3.197	Mitochondrion	*Daphnia pulex*	DQ340825
WTH001_0012_P06	-3.195	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0013_B24	-3.165	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0001_M08	-3.140	Mitochondrial genome	*Daphnia pulex*	AF117817

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Down-regulated genes (Cont.)				
WTH001_0014_K24	-3.138	Cuticle protein 7	*Blaberus craniifer*	P82120
IGU001_0013_F02	-3.136	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0001_J11	-3.039	DEAD-box RNA-dependent helicase p68	*C. auratus*	AY821682
WTH001_0007_L07	-3.022	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0031_D12	-3.021	39 ribosome-associated membrane protein	*O. fasciatus*	EF382747
IGU001_0021_H07	-3.006	Mitochondrion	*Daphnia pulex*	DQ340839
WTH001_0004_P21	-2.986	Cuticle protein CB7-like	*P. pelagicus*	EF102012
WTH001_0014_D22	-2.958	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0044_D10	-2.938	18S rRNA gene	*Daphnia magna*	AM490278
IGU001_0019_B07	-2.893	Mitochondrion	*Daphnia pulex*	DQ340832
WTH001_0010_P23	-2.796	Cuticle protein 7	*Blaberus craniifer*	P82120
IGU001_0033_A12	-2.772	Ubc D1 mRNA for ubiquitin-conjugating enzyme	*D. melanogaster*	X62575
WTH001_0001_I21	-2.759	Obstructor-A CG17052-RA	*D. melanogaster*	NM_134534
WTH001_0007_E11	-2.627	Peroxinectin	*P. monodon*	AF188840
IGU001_0030_G11	-2.613	Ribosomal protein L9	*A. franciscana*	ABC02755
WTH001_0014_K09	-2.598	Cuticle protein CB7-like	*P. pelagicus*	EF102012
IGU001_0045_A09	-2.578	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0013_A05	-2.574	dhb3 mRNA for haemoglobin	*Daphnia magna*	AB021137
IGU001_0014_E03	-2.562	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0015_D12	-2.463	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0019_C08	-2.459	Ribosomal protein S19	*A. monolakensis*	DQ886792
IGU001_0020_E06	-2.422	Thymosin	*Bombyx mori*	NM_001110348
IGU001_0051_H06	-2.400	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0012_G09	-2.234	GM2 ganglioside activator protein	*Danio rerio*	AAH92784
IGU001_0002_A03	-2.227	Mitochondrion	*Daphnia pulex*	DQ340839
WTH001_0005_G08	-2.210	Carboxylesterase	*S. exigua*	EF580101
IGU001_0032_H11	-2.142	GM2 ganglioside activator protein	*Xenopus laevis*	AAH74424
IGU001_0020_B04	-2.114	Mitochondrion	*Daphnia pulex*	DQ340820
IGU001_0054_F02	-2.013	Vesicle coat complex COPII GTPase subunit	*Aedes aegypti*	DQ440264
IGU001_0043_F09	-1.997	16S ribosomal RNA gene	*Daphnia magna*	DQ470575
IGU001_0043_C02	-1.955	Ribosomal RNA gene	*Daphnia magna*	AY921452
WTH001_0003_A23	-1.939	Ribosomal protein L28	*Sphaerius sp.*	CAJ17404
IGU001_0007_G01	-1.911	Cytochrome c oxidase subunit 1	*Daphnia pulex*	NP_008622
IGU001_0014_F05	-1.906	Receptor expression-enhancing protein 5	*Danio rerio*	AAH59545
IGU001_0038_H10	-1.903	Ribosomal protein S20	*O. mykiss*	CAC44156
IGU001_0050_B09	-1.897	Putative peptidyl-prolyl cis-trans isomerase E	*M. hirsutus*	EF070482
IGU001_0018_B12	-1.888	ATP synthase a chain	*Daphnia pulex*	Q95782
IGU001_0009_D11	-1.870	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0001_G20	-1.859	Similar to adenylosuccinate synthetase (predicted)	*Bos taurus*	NM_001099192
IGU001_0020_D04	-1.845	Mitochondrion	*Daphnia pulex*	DQ340832
IGU001_0031_D07	-1.841	Cytochrome b	*Daphnia pulex*	NP_008632
IGU001_0056_E06	-1.798	Ribosomal protein S25	*P. albinus*	CAH04344
IGU001_0017_B10	-1.758	Chymotrypsin B2	*L. vannamei*	CAA71673
WTH001_0012_A19	-1.749	Peroxinectin	*P. monodon*	AF188840
WTH001_0005_K21	-1.737	Fatty acid binding protein	*S. japonicum*	L23322
WTH001_0009_O15	-1.730	Cuticle structural protein post-ecdysial	*Tenebrio molitor*	S78003
WTH001_0012_A01	-1.716	Chymotrypsin 1	*L. vannamei*	X66415
IGU001_0028_H07	-1.662	Cuticle structural protein post-ecdysial	*Tenebrio molitor*	Q7M4D9
WTH001_0001_N05	-1.657	Elongation factor 1 alpha	*Bombyx mori*	NM_001044045
WTH001_0001_M13	-1.626	Chitin binding domain-containing protein	*A. franciscana*	EU072032
WTH001_0005_I15	-1.624	Fatty acid binding protein	*S. japonicum*	L23322
IGU001_0045_C04	-1.612	Mitochondrial genome	*Daphnia pulex*	AF117817
IGU001_0023_A03	-1.604	Peptidyl-prolyl cis-trans isomerase	*A. subalbatus*	EU207997
WTH001_0010_G24	-1.579	DD5 (structural constituent of cuticle)	*M. japonicus*	AB049147

CLONE ID	RATIO	GENE DESCRIPTION	SPECIES	ACCESSION
Down-regulated genes (Cont.)				
WTH001_0012_H23	-1.547	Fatty acid binding protein	*S. japonicum*	L23322
IGU001_0021_A10	-1.505	Heat shock protein HSP90	*D. klunzingeri*	Y17848
IGU001_0014_G11	-1.496	Annexin B10C	*A. gambiae*	XM_310252
WTH001_0004_L21	-1.432	Extracellular cyanophycinase (cphE)	*P. anguilliseptica*	AY065671
WTH001_0007_E08	-1.413	Similar to doughnut on 2 CG17559-PA (predicted)	*Apis mellifera*	XM_393673
WTH001_0003_H02	-1.388	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0005_P10	-1.376	Similar to apolipoprotein D (predicted)	*S. purpuratus*	XM_001181564
WTH001_0001_N02	-1.352	8S rRNA gene	*Daphnia magna*	AM490278
IGU001_0002_E11	-1.336	Mitochondrion	*Daphnia pulex*	DQ340832
IGU001_0011_E08	-1.299	Obstractor D	*T. castaneum*	NM_001080099
WTH001_0007_M04	-1.257	Small nuclear ribonucleoprotein D3	*Mus musculus*	NM_026095
IGU001_0024_G02	-1.256	Acidic p0 ribosomal protein	*D. cervinus*	AJ783862
IGU001_0043_D05	-1.228	Putative Rab7 mRNA	*O. nigricans*	AY725788
IGU001_0020_D03	-1.222	Palmitoyl-protein thioesterase 2	*Bos taurus*	NM_001035318
IGU001_0011_C05	-1.199	16S ribosomal RNA gene	*Daphnia magna*	AY921452
WTH001_0014_D19	-1.181	Trehalose transporter AgTRET1	*A. gambiae*	AB369548
WTH001_0009_C09	-1.165	Mitochondrial ribosomal protein L2 (nuclear gene)	*Bombyx mori*	NM_001044151
WTH001_0011_I11	-1.092	GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase	*D. melanogaster*	NM_137890
IGU001_0020_B05	-1.063	Mitochondrial genome	*Daphnia pulex*	AF117817
WTH001_0005_O23	-1.030	Fatty acid binding protein	*S. japonicum*	L23322
WTH001_0005_P14	-1.006	Similar to Headcase protein (predicted)	*Apis mellifera*	XM_001121077
IGU001_0019_C12	-1.001	Strain S11 mitochondrion	*Daphnia pulex*	DQ340832

FULL LIST OF SPECIES MATCHED IN HOMOLOGY SEARCH:

Vertebrates – *Bos taurus*; *Carassius auratus; Danio rerio*; *Homo sapiens*; *Mus musculus*; *Oncorhynchus mykiss*; *Solea senegalensis; Sus scrofa*; *Xenopus laevis*; *Xenopus tropicalis*.

Invertebrates – *Acyrthosiphon pisum*; *Aedes aegypti*; *Anopheles gambiae*; *Aplysina fistularis*; *Apis mellifera*; *Apriona germari*; *Argas monolakensis*; *Armigeres subalbatus*; *Artemia franciscana*; *Aurelia aurita*; *Blaberus craniifer; Bombyx mori*; *Caenorhabditis elegans*; *Corbicula fluminea*; *Daphnia magna*; *Daphnia pulex*; *Dascillus cervinus*; *Dendronephthya klunzingeri*; *Diaphorina citri*; *Drosophila melanogaster*; *Drosophila orena*; *Eurythoe complanata; Helicoverpa assulta*; *Hydra vulgaris*; *Lepeophtheirus salmonis*; *Litopenaeus vannamei*; *Lonomia obliqua*; *Lysiphlebus testaceipes*; *Maconellicoccus hirsutus*; *Marsupenaeus japonicus*; *Mytilus edulis*; *Neogonodactylus oerstedii*; *Oncometopia nigricans*; *Oncopeltus fasciatus*; *Ornithodoros parkeri*; *Pacifastacus leniusculus*; *Penaeus monodon*; *Platystomos albinus*; *Portunus pelagicus*; *Schistosoma japonicum*; *Sphaerius* sp.; *Spodoptera exigua*; *Strongylocentrotus purpuratus*; *Tachypleus tridentatus*; *Tenebrio molitor*; *Triatoma dimidiata*; *Tribolium castaneum*.

Bacteria – *Pseudomonas anguilliseptica*;

Capítulo VI

Considerações finais

O estudo das massas de água doce enquanto ecossistema, muito por força da sua escassez relativa e da relevância desse condicionalismo para as populações humanas, é central nos domínios fundamental e aplicado das ciências biológicas. Actualmente, a definição de ecossistema lêntico considera, não só a massa de água propriamente dita, mas também a bacia de drenagem que a ela está intimamente ligada enquanto zona mediadora da chegada de substâncias orgânicas e inorgânicas ao sistema aquático (Wetzel 1993). De facto, as condições ambientais impostas pelos fluxos de entrada de substâncias diversas na massa de água estão directamente relacionadas com a dinâmica estrutural do biota aquático – a introdução de factores indesejáveis no sistema poderá ter consequências, por exemplo, ao nível da sucessão natural das comunidades.

O trabalho desenvolvido na presente dissertação foi enquadrado nesta problemática considerando que: (i) a descarga de nutrientes, via bacia de drenagem, no compartimento aquático estimula directamente a produtividade do lago através da promoção do crescimento fitoplanctónico (e.g., Smith et al. 1999, Gulati & Van Donk 2002, Carpenter 2005), o que altera decisivamente as condições alimentares dos cladóceros, influenciando a sua dinâmica populacional; (ii) a crescente utilização de pesticidas em terrenos agrícolas adjacentes a sistemas lênticos incrementa a probabilidade de estes compostos chegarem ao sistema aquático ainda com potencial tóxico para afectarem a sobrevivência ou a reprodução dos cladóceros, induzindo, de uma forma indirecta, alterações eventualmente dramáticas na estrutura trófica da zoocenose (e.g., Hanazato 1998, 2001).

Neste contexto, os quatro capítulos anteriores desta dissertação registam a abordagem de duas linhas gerais de investigação. Por um lado, produziram-se evidências experimentais da plasticidade genotípica das populações de *Daphnia* nas respostas a estímulos ambientais de natureza diferente (Capítulos II, III e IV), plasticidade essa que poderá ser um factor chave na conceptualização da extensão da tolerância dos sistemas aquáticos à alteração das condições ambientais. Por outro, considerando cenários de exposição aguda e crónica e avaliações a diferentes níveis de organização biológica, analisou-se a toxicidade de dois pesticidas para diferentes populações de *Daphnia*, entendidas enquanto populações não-alvo desses mesmos pesticidas; foram detectadas importantes vias de acção tóxica traduzidas em efeitos deletérios ao nível da sobrevivência e da reprodução, potencialmente condicionadores do eventual sucesso das populações sob condições ambientais reais semelhantes. A concepção destas duas directrizes de

trabalho tem as suas raízes nos procedimentos que têm sido propostos, ao nível dos círculos científicos e decisores, para a interpretação e avaliação do risco que os compostos com potencial de contaminação, usados na protecção da produção agrícola, representam para os ecossistemas aquáticos (e.g., CE 1991, EC 2002). A regulamentação de procedimentos de avaliação de risco tende a adoptar metodologias normalizadas que, por natureza, não têm em conta a variabilidade associada a factores extrínsecos à análise.

De facto, os documentos da União Europeia que regulam a colocação de produtos fitofarmacêuticos no mercado e orientam a respectiva avaliação de risco recomendam a utilização de *Daphnia magna* como bioindicador, em testes normalizados de toxicidade aguda e crónica (CE 1991, EC 2002), por esta ter sido considerada a espécie mais sensível a contaminantes orgânicos, de entre uma amostra abrangente de diversos invertebrados aquáticos, num estudo de revisão por Wogram & Liess (2001). Nesta revisão demonstra-se que *D. magna* é bastante menos tolerante à exposição aguda a contaminantes orgânicos do que, por exemplo, os gastrópodes, os oligoquetas, os tricópteros ou mesmo os copépodes; no entanto, a espécie é tendencialmente mais tolerante do que os outros Cladocera considerados no estudo (as espécies em causa não são discriminadas). Os dados registados nos capítulos II, III e IV apontam, à semelhança de evidências obtidas em estudos anteriores (Gliwicz 1990, Baird et al. 1991, Epp 1996, Antunes et al. 2003, 2004, Marques et al. 2004a,b) para a existência de diferenças significativas na tolerância de diferentes espécies de *Daphnia*, quer a alterações nas condições ambientais naturais (i.e. disponibilidade alimentar), quer à exposição a contaminantes orgânicos (i.e. pesticidas e outros xenobióticos), mesmo quando a comparação considera espécies taxononomicamente muito próximas ou genótipos distintos da mesma espécie. Adicionalmente, ao considerar-se a disponibilidade alimentar na avaliação da toxicidade dos pesticidas, evidenciou-se o papel da dinâmica energética de *Daphnia* na resposta ao *stress*; ou seja, em concordância com conclusões de estudos anteriores [revistas por Heugens et al. (2001) e Smolders et al. (2005)], a disponibilidade de itens alimentares – e o consequente balanço energético do organismo – revelou-se determinante da capacidade de resposta das populações de *Daphnia* à exposição aos tóxicos, condicionando, portanto, a sua tolerância a esse mesmo tóxico. Assim, as observações efectuadas no decorrer deste trabalho sustentam a hipótese de uma eventual adição de incerteza, aquando da aplicação estrita de recomendações e testes normalizados na avaliação do risco dos xenobióticos para o ecossistema aquático. Em análises de risco aplicadas a sistemas particulares deverá admitir-se a necessidade de ajustar metodologias às condições concretas do sistema, por exemplo, utilizando espécies indígenas e equacionando

potenciais factores extrínsecos ao objecto de análise, mas que manifestamente possam interagir com ele no cenário ecológico real.

Sob uma perspectiva mais específica, esta dissertação também deve ser entendida como uma abordagem aplicada aos mecanismos que gerem a resposta de *Daphnia* ao *stress*, de génese natural ou artificial, independentemente do papel da variabilidade genotípica nessa gestão, acima discutido. A análise da influência da disponibilidade de alimento na história de vida e no *fitness* das populações de *Daphnia* permitiu confirmar evidências, já registadas na literatura (e.g. Guisande & Gliwicz 1992, Boersma 1995, Trubetskova & Lampert 1995, Polishchuk & Vijverberg 2005), que demonstram a elevada sensibilidade dos cladóceros às flutuações nos recursos alimentares. Estes organismos adaptam-se às variações que ocorrem nas condições alimentares, entendidas aqui como variações na concentração de alimento edível, ajustando as estratégias de alocação energética; ou seja, considerando o potencial energético que é possível obter sob um determinado nível de disponibilidade de recursos alimentares, *Daphnia* investe selectivamente nos processos fisiológicos centrais (reprodução, crescimento, manutenção e, eventualmente, armazenamento), respeitando um equilíbrio dinâmico cujo fim último é sempre o de assegurar a longevidade e a consequente capacidade reprodutiva a longo termo (Kooijman 1986, McCauley et al. 1990, Polishchuk & Vijverberg 2005, Rinke & Vijverberg 2005). Quando a disponibilidade de recursos é total, *Daphnia* investe fundamentalmente na maximização da reprodução, sendo que, à medida que os recursos alimentares começam a escassear, as prioridades na alocação de energia passam a favorecer o crescimento somático e, em condições extremas de pobreza na aquisição de energia, a manutenção da condição fisiológica, enquanto estratégia de sustentação basal.

A dinâmica energética parece aliás ser um factor condicionante da resposta de *Daphnia* a compostos potencialmente tóxicos (*vide* considerações anteriores). Os efeitos da exposição quer ao herbicida Propanil, quer ao insecticida Metomil (capítulos II e IV), revelaram-se variáveis quanto à amplitude, consoante a disponibilidade de recursos alimentares prevalente nos tratamentos, isto é, consoante o potencial energético dos organismos para responderem a essa mesma exposição. A base conceptual desta observação é relativamente lógica porque deriva do princípio básico de que lidar com um estímulo tóxico envolverá custos energéticos adicionais, relativamente aos envolvidos no metabolismo de rotina, com as consequentes alterações nas prioridades estratégicas de alocação de energia para os vários compartimentos fisiológicos (Congdon et al. 2001, Knops et al. 2001). Estes custos podem ser associados fundamentalmente com os mecanismos de metabolismo

secundário, que possam ser activados no âmbito de processos de destoxificação ou de reparação de danos, sendo assim determinantes na dinâmica da alocação de energia em *Daphnia* (e.g., Knops et al. 2001). Muito embora mais visível na exposição de *Daphnia* ao herbicida, a resistência a ambos os tóxicos, expressa em termos do sucesso registado nos parâmetros reprodutivos e populacionais analisados, foi tendencialmente maior quando a disponibilidade alimentar era menor. Embora este seja um padrão aparentemente contraditório, foi já observado por outros autores (Folt et al. 1999, Polishchuk & Vijverberg 2005, Smolders et al. 2005), tendo sido atribuído a alterações compensatórias no mecanismo de alocação de energia. Ou seja, sob condições alimentares limitantes, a prioridade no investimento do potencial energético é sempre a manutenção da condição fisiológica, logo a capacidade de resistência ao stress tóxico tende a aumentar; assim, ao encontrar-se sob limitação de recursos, toda a energia disponível é alocada para o compartimento fisiológico a que estão associados os processos que permitem a *Daphnia* resistir ao *stress* tóxico, com isso aumentando a probabilidade de sobrevivência (Polishchuk & Vijverberg 2005, Smolders et al. 2005).

Os pesticidas, enquanto contaminantes ambientais, diferem de todos os outros xenobióticos pelo facto de serem intencionalmente colocados no ambiente para combater pragas prejudiciais à produção agrícola. Mais ainda, são elaborados especificamente para provocar danos nos organismos-alvo através de mecanismos bioquímicos que, em geral, são comuns a muitos outros organismos estabelecidos nos locais de aplicação. Tipicamente, a avaliação da toxicidade de xenobióticos é centrada nas respostas dos organismos ao nível individual e populacional (Neumann & Galvez 2002). Tendo sido esta a abordagem seguida nos capítulos III e IV, o capítulo V foi concebido com base numa metodologia diferente, que facilitou a análise dos mecanismos sub-celulares subjacentes à toxicidade dos pesticidas para *Daphnia*. A integração de ferramentas de genómica em estudos ecotoxicológicos é um processo promissor, que poderá possibilitar o acesso a informação relevante sobre os mecanismos associados à toxicidade e, portanto, constituir uma plataforma de obtenção de dados fundamentais na avaliação do risco que os contaminantes representam para organismos e ecossistemas não-alvo (Neuman & Galvez 2002, Snape et al. 2004, Robbens et al. 2007). No contexto específico da presente dissertação, a utilização de uma destas ferramentas (*cDNA microarrays* – capítulo V) permitiu, não só confirmar que a exposição aos pesticidas induziu alterações relevantes ao nível do metabolismo energético de *Daphnia*, como também evidenciou a activação de uma série de vias e processos fisiológicos associadas à resposta do organismo ao estímulo tóxico (e.g., biossíntese de proteínas, ecdise, síntese de hemoglobina,

degradação de xenobióticos). Adicionalmente, e confirmando o potencial da técnica advogado por outros autores (e.g., Neuman & Galvez 2002, Snape et al. 2004), foi possível sinalizar alguns processos moleculares que poderão eventualmente ser usados para desenvolver biomarcadores específicos de exposição aos tóxicos em questão, sendo esta uma linha de trabalho em aberto, com vasto potencial de exploração futuro.

Mais do que um estudo abrangente sobre as variações operadas por populações de distintas de *Daphnia* em resposta a diferentes estímulos de *stress*, esta dissertação pretendeu reunir informação e gerar evidências experimentais novas e úteis acerca dos processos que podem regular essa mesma resposta. Utilizando a disponibilidade alimentar e a exposição a xenobióticos como base de trabalho e tentando responder a questões específicas associadas a diferentes metodologias experimentais, espera-se, no geral, ter contribuído para uma concepção mais realista da extensão dos impactos da variação das condições ambientais na dinâmica das populações de cladóceros e, em última instância, no ecossistema aquático.

REFERÊNCIAS

Antunes SC, Castro BB & Gonçalves F (2003). Chronic responses of different clones of *Daphnia longispina* (field an ephippia) to different food-levels. *Acta Oecologica* 24: S325-S332.

Antunes SC, Castro BB & Gonçalves F (2004). Effect of food level on the acute and chronic responses of daphnids to lindane. *Environmental Pollution* 127: 367-375.

Baird DJ, Barber I, Bradley M, Soares AMVM & Calow P (1991). A comparative study of genotype sensitivity to acute stress using clones of *Daphnia magna* Straus. *Ecotoxicology and Environmental Safety* 21: 257-265.

Boersma M (1997b). Offspring size in *Daphnia*: does it pay to be overweight? *Hydrobiologia* 360: 79-88.

Carpenter SR (2005). Eutrophication of aquatic ecosystems: Bistability and soil phosphorous. *Proceedings of the National Academy of Sciences of the USA* 19: 10002-10005.

CE (1991). Directiva 91/414/EEC do Conselho de 15 de Junho de 1991 relativa à colocação dos produtos fitofarmacêuticos no mercado. Jornal Oficial das Comunidades Europeias L230 de 19.08.1991: 1-32.

Congdon JD, Dunham AE, Hopkins WA, Rowe CL & Hinto TG (2001). Resource allocation-based life histories: a conceptual basis for studies of ecological toxicology. *Environmental Toxicology and Chemistry* 20: 1698-1703.

EC (2002). Guidance Document on Aquatic Ecotoxicology – in the context of the Directive 91/414/EEC. European Comission – Heath & Consumer Protection Directorate-General.

Epp GT (1996). Clonal variation in the survival and reproduction of *Daphnia pulicaria* under low-food stress. *Freshwater Biology* 35: 1-10.

Folt CL, Chen CY, Moore MV & Burnaford J (1999). Synergism and antagonism among multiple stressors. *Limnology and Oceanography* 44(3 part2): 864-877.

Gliwicz ZM (1990). Food thresholds and body size in cladocerans. *Nature* 343: 638-640.

Guisande C & Gliwicz ZM (1992). Egg size and clutch size in two *Daphnia* species grown at different food levels. *Journal of Plankton Research* 14: 997-1007.

Gulati RD & Van Donk E (2002). Lakes in the Netherlands, their origin, eutrophication and restoration: state-of-the-art review. *Hydrobiologia* 478: 73-106.

Hanazato T (1998). Response of a zooplankton community to insecticide application in experimental ponds: a review and the implications of the effects of chemicals on the structure and functioning of freshwater communities. *Environmental Pollution* 101: 361-373.

Hanazato T (2001). Pesticide effects on freshwater zooplankton: an ecological perspective. *Environmental Pollution* 112: 1-10.

Knops M, Altenburger R & Segner H (2001). Alterations of physiological energetics, growth and reproduction of *Daphnia magna* under toxicant stress. *Aquatic Toxicology* 53: 79-90.

Kooijman SALM (1986). Population dynamics on the basis of budgets. *In*: JAJ Metz. & O Diekman (eds.), The dynamics of physiologically structured populations. Springer Verlag, Berlin, pp. 266-297.

Marques CR, Abrantes N & Gonçalves F (2004a). Life-history traits of standard and autochtonous cladocerans: I. Acute and Chronic effects of acetylsalicylic acid. *Environmental Toxicology* 19: 518-526.

Marques CR, Abrantes N & Gonçalves F (2004b). Life-history traits of standard and autochtonous cladocerans: II. Acute and chronic effects of acetylsalicylic acid metabolites. *Environmental Toxicology* 19: 527-540.

McCauley E, Murdoch WW, Nisbet RM & Gurney WSC (1990). The physiological ecology of *Daphnia*: development of a model of growth and reproduction. *Ecology* 71: 703-715.

Neumann NF & Galvez F (2002). DNA microarrays and toxicogenomics: applications for Ecotoxicology? *Biotechnology Advances* 20: 391-419.

Polishchuk LV, Vijverberg J (2005). Contribution analysis of body mass dynamics in *Daphnia*. *Oecologia* 144: 268-277.

Rinke K & Vijverberg J (2005). A model approach to evaluate the effect of temperature and food concentration on individual life-history and population dynamics of *Daphnia*. *Ecological Modelling* 186: 326-344.

Robbens J, Van der Ven K, Maras M, Blust R & de Coen W (2007). Ecotoxicological risk assessment using DNA chips and cellular reporters. *TRENDS in Biotechnology* 25: 460-466.

Smith VH, Tilman GD & Nekola JC (1999). Impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution* 100: 179-196.

Smolders R, Bailieul M & Blust R (2005). Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. *Aquatic Toxicology* 73: 155-170.

Snape JR, Maund SJ, Pickford DB & Hutchinson TH (2004). Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial Ecotoxicology. *Aquatic Toxicology* 67: 143-154.

Trubetskova I & Lampert W (1995). Egg size and egg mass of *Daphnia magna*: response to food availability. *Hydrobiologia* 307: 139-145.

Wetzel RG (1993). *Limnologia* (trad. MJ Boavida). Fundação Calouste Gulbenkian, Lisboa, 919p.

Wogram J & Liess M (2001). Rank ordering of macroinvertebrate species sensitivity to toxic compounds by comparison with that of *Daphnia magna*. *Bulletin of Environmental Contamination and Toxicology* 67: 360-367.